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THE UNIVERSITY OF ALBERTA

LOCALIZATION OF SOME GLANDULAR KALLIKREINS.

By

Ⓒ BRUNO J. M. MARANDA

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

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ABSTRACT.

Immunofluorescence microscopy was used to attempt to localize three kallikreins, namely, those of the guinea-pig coagulating gland, cat submandibular gland and dog kidney. In addition, the kallikrein in the guinea-pig submandibular gland was localized using the antibody to coagulating gland kallikrein; also, using the antibody to cat submandibular gland kallikrein, the latter was localized in the parotid gland of the cat.

Specific fluorescence in the coagulating gland was present diffusely in all secretory cells lining the lumen. In contrast, kallikrein in the salivary glands of the cat and guinea-pig was located at the luminal border of striated and some larger duct cells. Other structures such as acinar and demi-lune cells, or interstitium, showed no significant fluorescence.

Good correlation was also observed between the amount of specific fluorescence and kallikrein concentrations present in the submandibular gland after procedures which progressively deplete the gland of kallikrein, namely sympathetic nerve stimulation or chronic duct ligation.

We also attempted to correlate specific immunofluorescence, kallikrein content, and secretory granules of the duct system by combined electron-microscopic studies. Our results suggest that this enzyme is located in small granules of the entire duct system, the smaller striated ducts in particular.

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INTRODUCTION

1. Kallikrein-Kinin System.

1.1 Kallikreins.

Kallikreins (or Kininogenases) (EC 3.4. 21.8.) are serine proteases releasing small peptides, kinins, from kininogens -their substrates- found in plasma and lymph. The actions of kallikrein are mainly caused by the biologically active kinins they release. This system is thus very similar to the renin-angiotensin system where a proteolytic enzyme also cleaves a precursor to produce a biologically active peptide.

Kallikreins were discovered more than fifty years ago when Frey (1926) observed that dog and human urine caused a marked fall in arterial blood pressure in the dog after intravenous injection. The same observations had been made earlier by Abelous and Barbier (1909) and Pribram and Hernnheiser (1920) but they did not pursue further their investigations. Frey, however, found that the hypotensive action was caused by a non-dialysable, heat sensitive compound, thus not being histamine, as had been suggested to him. The same, or a similar, substance was shown also to be present in blood (Frey and Kraut, 1928; Kraut, Frey and Werle, 1933), pancreas (Kraut, Frey and Werle,1930) and salivary glands (Werle and von Roden, 1936). These researchers believed that this hypotensive substance was formed in the pancreas, released in the blood circulation and eliminated by the kidneys. They therefore named it kallikrein, from *kallikreas,* the Greek term for pancreas. Frey and co-workers also found that blood and pancreatic kallikrein, in contrast to urinary kallikrein, was present in an inactive form, and that activation could be achieved by change in pH, by organic solvents (Werle, 1934; 1936) and by enzymes like pepsin and trypsin (Kraut et al, 1933; (Werle and Urhahn, 1940). Since its action was similar to a vasodilator hormone, it was also termed "KREISLAUF-hormon", which means circulatory hormone.

Another important fact about the kallikrein system was revealed in 1937 by Werle and co-workers (Werle, Götze and Keppler, 1937) when they found that kallikrein released a potent smooth muscle contracting substance from inactive precursor in plasma. This substance was called "Substanz DK" or "Darmkontrahierende Substanz" i.e. Gut-contracting substance. A year later, Werle and Berek (1938) changed the name of Substanz DK to kallidin and its precursor, the substrate for kallikrein, kallidinogen.

Another decade later, Brazilian researchers (Rocha e Silva, Beraldo and Rosenfeld, 1949) discovered a hypotensive and smooth muscle stimulating factor, released from plasma globulin by snake venoms and by trypsin, which they called bradykinin. There was great similarity between the kallikrein-kallidinogen-kallidin system and the trypsin-bradykinogen-bradykinin system. Subsequently, Schachter and co-workers (Jaques and Schachter, 1954; Schachter and Thain, 1954; Holdstock, Mathias and Schachter, 1961; Bhoola, Calle and Schachter, 1961) introduced the generic term, kinin, after discovering peptides in free form in wasp and hornet venom, which were very similar to bradykinin and kallidin in pharmacological and chemical properties. The term kinin was proven to be prophetic since it was later shown that many of these peptides had a major common amino acid sequence (see Schachter, 1969; Pisano, 1975). The terms kininogenase and kininogen were also introduced by Schachter's group when it was discovered that the accessory sex gland of the guinea-pig contained a kallikrein like enzyme (Bhoola, May May Yi, Morley and Schachter, 1962). In recent investigations (see Pisano, 1975; Schachter, 1969; Vogel, Werle and Zickgraf-Rudel, 1970) it has been shown that all kallikreins are not identical molecules but are nonetheless similar enough to be classified under the same generic name.

Kallikreins or kinonogenases are classified as serine proteases which include enzymes like trypsin, chymotrypsin, elastase, thrombin and fibrinolysin(Stroud, 1974), and may be present in multiple forms in some tissues. These multiple forms of kallikreins have been observed in rat urine (Nustad and Pierce, 1974), rat submandibular gland (Ekfors, 1967), pig pancreas (Fiedler, Hirschauer and Werle, 1975) and submandibular gland (Fiedler, Muller and Werle, 1970). There seem to be some differences in the specificity of the various kallikreins for their substrates.

Glandular kallikreins release the decapeptide kallidin (or lysyl-bradykinin) while plasma kallikrein and some other serine proteases like trypsin, fibrinolysin, and enzymes found in snake venoms yield the nonapeptide bradykinin (Schachter, 1969; Pisano, 1975). Glandular kallikreins are relatively small glycoproteins. their molecular weight varying from 25 to 40,000 (Schachter, 1969; Pisano, 1975; Suzuki, Takahaski, Kmiya, Horsuchi and Nagasawa, 1972; Moriwaki, Nuki, Fujimoto and Moriya, Fujimoto and Ueki, 1976). Plasma kallikreins present in multiple forms in human plasma as prekallikreins are much larger, having a molecular weight of over 100,000 (Colman, Mattler and Sherry, 1969; Sampaio, Wong and Shaw, 1974).

Since all the kallikreins hydrolyse various synthetic amino acids esters, this hydrolysis is the basis of chemical assays, such as the method described by Trautschold (1970) using α-N-benzoyl-L-arginine ethyl ester (BAEe) as substrate. The bio-assay for kininogenase activity determination is based on the release of kinin, measuring its hypotensive action *in vivo* or its contractile action on smooth muscle *in vitro* (Moriwaki and Schachter, 1971).

1.1.1 Glandular kallikreins.

As mentioned above, kallikreins have been found in many tissues of various animals. Kallikrein was found nearly fifty years ago in the pancreas (Kraut et al., 1930) but has been localized in that tissue only recently (Proud, Bailey, Orstavik and Nustad, 1977; Dietl, Kruck and Fritz, 1978). Investigations concerning various glandular kallikreins are described below.

1.1.1.1 Pancreatic Kallikrein.

Kallikrein is present in the pancreas as an inactive precursor (prekallikrein) which is secreted into the duodenum with other digestive enzymes and where it is activated by trypsin and enterokinase (Werle, 1955; Werle, Forell and Maier, 1955). The function of kallikrein in the pancreas is believed to be exocrine (Forell, 1960; Schachter, 1962; Fiedler, 1976) since its concentration in the secretion parallels that of trypsin and lipase and since its concentration is reduced like that of other digestive enzymes after pancreatic duct ligation. From the fact that kallikrein is reduced by duct ligation, it was hypothesized that kallikrein originates in exocrine cells. Also its concentration was not affected by alloxan treatment which affects the β - cells of the islets of Langerhans (Forell, 1960). The localization of kallikrein in the acinar cells of the pancreas, of both the rat and the pig, was recently demonstrated by immunofluorescence techniques (Proud et al.,1977; Dietl et al., 1978). This localization differs from that of kidney and salivary glands (see later).

1.1.1.2 Intestinal Kallikrein.

The wall of the mammalian large and small intestine contains a substance which appears to be a kininogenase due to its hypotensive action (Werle, 1960; Werle and Vögel, 1961). This kallikrein is present mostly in inactive form and can be activated by trypsin.

1.1.1.3 Kidney and Urinary Kallikrein.

A kallikrein-like substance was first discovered in dog and human urine (Abelous and Barbier, 1909; Frey, 1926). It had been postulated (Werle, 1960; Carvalho and Diniz, 1964) that the kidney was the source of urinary kallikrein, since after experimental damage to the proximal tubules, kallikrein was no longer found in an active form in the urine (Werle and Vögel, 1960) but replaced by pre-kallikrein which could normally be found in kidney homogenates. This selective damage suggested that kallikrein might in fact be "activated" or excreted by the proximal tubules; however, its recent localization in rat by immunofluorescence (Orstavik, Nustad, Brandtzaeg and Pierce, 1976) showed that kallikrein is present in the distal tubular cells and that there is cross-antigenicity between urinary and renal kallikrein. On the other hand, Dietl and co-workers (1978) suggested that urinary kallikrein might be simply pancreatic and/or submandibular kallikrein filtered by the kidneys (and absorbed by them) and that further careful investigation would be necessary to answer the question whether there is a kidney specific kallikrein.

1.1.1.4 Salivary Kallikrein.

Since the first observations of Werle and co-workers (Werle and von Roden, 1936) extensive work had been done on this source of kallikrein. This will be discussed in detail in section 2 of the Introduction.

1.1.1.5 Accessory Sex Gland Kallikrein.

Kallikrein has been described in gland extract of guinea-pig's coagulating and prostate glands (Bhoola et al., 1962; Moriwaki and Schachter, 1971). Although the substrate is present in plasma of guinea-pig and other mammals, the enzyme itself was not found in the prostate glands of man, dog, cat and rabbit.

1.1.2 Other Kininogenases.

Trypsin (Rocha e Silva et al., 1949) and plasmin, as discovered by Beraldo (1950) and Schachter (1956), are the best known among the other proteolytic enzymes which release kinins. Following the original observation of Rocha e Silva (1949) concerning the presence of kinin releasing enzyme in snake venom, many other workers found the same to be true for many snake species (Deutsch and Diniz, 1955; Habermann, 1963; Iwanaga, Omori, Ochima and Suzuki, 1965; Iwanaga, Sato, Mizushima and Suzuki, 1965) suggesting that this kininogenase corresponded to the salivary kallikrein of the snake.

Bacterial kininogenases were also found in *Clostridium histolyticum* (Prado, Monier, Prado and Fromagyot, 1956) and in *B. subtilis* (Nagarse) (Huggins and Thampi, 1967; Prado, Prado and Jurkiewicz,1964). This bacterial enzyme released a kinin from horse serum but had little or no effect on BAEe. Kininogenases were reported to be present in other tissues or body fluids such as sweat (Fox and Hilton, 1958; Werle, 1968), bronchial mucosa (Havez, 1966; Havez, De Goud, Roussel, Viosin, Biserte and Gernez-Rieux, 1966), cerebro-spinal fluid during neurological disturbances (Ramos, Chapmar and Ramos, 1963), and in brain, lymph glands and spleen (Werle and Vögel, 1961).

Fritz and co-workers (Fritz, 1975; Palm and Fritz, 1975) showed that human and mammalian spermatozoa contained a trypsin like enzyme, likely located in the acrosome and therefore named acrosin. Recent investigations (Schill, Schleuning, Fritz, Wendt and Heimburger, 1975; Garner and Easton, 1976; Morton, 1975) showed that acrosin is located at the inner acrosomal membrane but that it is not similar to trypsin (Allen, 1976).

Kininogenases are thus present in various tissues and body fluids. This wide distribution may suggest a general rôle, which has yet to be defined.

1.2 Kinins.

It is believed that kinins are responsible for the main actions of the kininogenases. Werle et al., (1937) first observed that kallikrein released a substance from serum that caused contraction of the intestinal smooth muscle. Rocha e Silva (1949) observed the same for trypsin and snake venoms. Schachter and Thain (1954) discovered substances in a free form, in wasp venom, which had characteristics similar to the substances previously discovered by the above mentioned researchers and they introduced the generic term, kinin.

1.2.1 Naturally occurring kinins and their properties.

As described in recent reviews (Schachter, 1969; Pisano, 1975) all known kinins have structure which are very closely related, e.g. the three mammalian kinins are bradykinin, lys-bradykinin (or kallidin) and Met-lys-bradykinin (or Met-kallidin). In all active kinins, the amino-acid sequence of bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is found except that amino-acids may be added at the carboxyl or amino end of the chain. The only exceptions are thr-bradykinin (Ishikawa, Yasuhara, Nakajima and Tachibana, 1974), val-thr- bradykinin (Nakajima, 1968), polisteskinin-R and renakinin-R (Ishikawa et al., 1974) in which the serine in position 6 is replaced by threonine.

Kinins have a number of pharmacological properties in common, such as a hypotensive effect, contraction of isolated smooth muscle preparation but relaxation of rat duodenum, increase in capillary permeability and pain production when applied to a blister base on human skin. Some of their recently discovered effects, for example, stimulation of prostaglandin synthesis, cell proliferation and enhancement of sperm motility, will be discussed in greater detail later in the introduction.

Bradykinin. kallidin and met-Lys-bradykinin are qualitatively similar by their pharmacological properties but differ quantitatively (Erdös, 1966; Frey et al., 1968; Schachter and Morley, 1964). For example, bradykinin is much more potent than kallidin and Met-Lys-bradykinin on the isolated guinea-pig ileum but the order is reversed in lowering arterial blood pressure or in vascular permeability tests (Reis, Okino and Rocha e Silva, 1971). Evidence shows that bradykinin is released from plasma kininogen by trypsin, plasma kallikrein and snake venoms while kallidin is the result of the action of the glandular kallikreins (Habermann, 1963; Kato, Nagasawa and Suzuki, 1967; Webster and Pierce, 1963). Some exceptions are snake venom from *Agkistrodon contortix* (Webster and Pierce, 1963) which releases both bradykinin and kallidin, and pancreatic kallikrein which releases Met-Lys-Bradykinin (Habermann and Miller, 1966). However, it should not be said that one kininogenase releases only one kind of kinin from one kind of substrate. There seem to be some flexibility in the kallikrein-kinin system, the specific kinin being released possibly varying with the nature of the substrate on which the enzyme is acting (Kato et al., 1967). Kinins were first described in free form in wasp venom (Schachter and Thain, 1954) but observed since then in many other tissues or secretions like amphibian skin (Anastasi, Bertaecini and Erspamer, 1966; Erspamer and Anastasi, 1966; Erspamer and Melchioni, 1973), yellowjacket and hornet venoms (Pisano, 1968; Bhoola et al., 1961; Yoshida and Pisano, 1974), reptile plasma (Erdös, Miwa and Graham, 1967) and human urine (Werle and Erdös, 1954).

Again, as with kininogenases, there is a wide distribution of kinins, suggesting an important but not yet identified biological rôle.

1.2.2 Kinin inhibitors and potentiators.

No specific drug which specifically blocks the action of kinin has yet been found. Many analogs have been prepared (Stewart, 1968; Schröder, 1970) but only non-competitive inhibitors which also block the action of histamine and/or serotonin on isolated smooth muscle have been described (see Rocha e Silva, 1970). The pain and inflammatory action of kinins can be reduced by analgesic drugs like aspirin but their inhibition of kinin action is believed to be due to their effect on the synthesis of prostaglandins which are thought to be the mediators in some actions of kinins (Palmer, Piper and Vane, 1973; McGiff, Termagno, Malik and Lonigro, 1972). The effect of many kinin potentiators appears to be due to their inhibition of the kininases, those enzymes responsible for the break down and inactivation of kinins. These potentiators include chelating agents like ethylenediamine tetraacetate (EDTA) (Bishop and Margolis, 1963), cysteine (Erdös, 1962) and mercaptoethanols, which are metal binding agents and bind the metal co-factor of these kininases (Schachter, 1969; Erdös and Yang, 1970). Small peptides have also been found to be kinin potentiators. They include fibrinopeptides (Gladner, Murtaugh, Folk and Laki, 1963; Gladner, 1966), trypsin-generated peptides (Hamberg, Elg and Stelluagen, 1968; Aarsen, 1968), peptides isolated from snake venoms (Ferreira, Rocha e Silva, 1965; Kato and Suzuki, 1968; Ferreira, Bartelt and Greene, 1970; Ondetti, Williams, Sabo, Pluscec, Weaver and Kocy, 1971) and synthetic peptides (Freer and Stewart, 1973; Stewart, Freer and Ferreira, 1973). These kinin potentiators are useful analytical tools of physiological processes and might also eventually be useful in therapy. They are also known as BPFs, Bradykinin-Potentiating-Factors (Ferreira and Rocha e Silva, 1965).

1.3 Kininogens and Kininases.

1.3.1 Kininogens.

The first kininogen preparation was made by Werle and co-workers (Werle et al., 1937) by heating plasma or serum for 1 to 3 hours at 60°C. It has since been shown that kininogens are acidic glycoproteins found in mammalian plasmas (Habermann,1970; Pierce, 1970) and that more than one kininogen is present in one plasma (Vogt, 1966). Two functionally different kininogens, differing both in molecular weight and susceptibility to kallikreins, were isolated from various mammalian plasmas (Jacobsen, 1966 a,b; Jacobsen and Kritz, 1967; Yano, Kato, Nagasawa and Suzuki, 1967 a,b: Henriques, Kauritchera, Kuznetsova and Astrakan, 1967; Komiya, Kato and Suzuki, 1974 c; Han, Komiya, Iwanaga and Suzuki, 1975). These are designated as high-molecular weight (H M W) and low molecular weight (L M W) kininogens, the molecular weight varying from 66,000 to 200,000 for H M W and from 48,000 to 57,000 for L M W. The kinin moiety is in the internal portion of the chain in the H M W kininogen, while located at the carboxyl terminal end of the chain for the L M W form. The H M W and L M W kininogens appear to be immunochemically identical (Komiya, Kato and Suzuki, 1974, a,b,c). All kininogens appear to release bradykinin, kallidin or Met-Lys-bradykinin when incubated with the appropriate kininogenases. The determination of the amount of kinin released is generally based on the method of Diniz and Carvalho (1963) which employs trypsin to release bradykinin from kininogen in plasma (acid-treated and heated to destroy kininases).

1.3.2 Kininases.

The first kinin-inactivating enzyme was discovered by Werle and co-workers when they investigated the release of kinin by kallikrein (Werle et al., 1937). It was later found by the same group (Werle and Hambuechen, 1943) and by Schachter (1960) that sera and lymph of various mammals inactivated kinin. The presence of kininases was also demonstrated in various tissues (Erdös and Yang, 1970; Erdös, 1971) and their properties and specificities established. A carboxypeptidase, (previously known as carboxypeptidase N, arginine carboxypeptidase or anaphylatoxin inhibitor) and which hydrolysed the Phe^8-Arg^9 bond of bradykinin was purified and named kininase I (Erdös, 1971; Oshima, Kato and Erdös, 1974). Another enzyme, kininase II, found in plasma, lung and kidney, and similar to the angiotensin I - converting enzyme or peptidyldipeptide hydrolase, was shown to split the Pro^7-Phe^8 bond of bradykinin, releasing the dipeptide Phe-Arg (Erdös, 1970, 1975; Nakajima, Oshima, Yeh, Igic and Erdös, 1973; Oshima, Gecse and Erdös, 1974). Other kininases have been described in lysed red cells (Erdös, 1970) and brain (Comargo, Shapanka and Greene, 1973). It thus appears that kininases are present in almost all organs and probably inactivate locally generated kinins or circulating ones. It should be noted that hydrolysis of any of the peptide bonds inactivates bradykinin and that kininases are not specific for kinins only. Many different kininases can thus inactivate kinins and a kininase can act on various substrates.

1.4 Physiological and pathological significance of the kallikrein-kinin system.

Due to their indirect action by the kinins they release or by their own actions, the kallikreins may be implicated in many pathological and physiological mechanisms. Some of these are considered below.

1.4.1 Glandular secretion and functional hyperaemia.

Since kallikreins are abundant in exocrine glands and kinins are potent vasodilators, one of the first physiological rôle proposed for the kallikrein-kinin system was that of functional vasodilatation (Hilton, 1960; Hilton and Lewis, 1955, a,b, 1956, 1958; Lewis, 1958, 1959, 1962, 1967). This proposal has however been challenged, mostly by Schachter and co-workers (Bhoola et al., 1962; Bhoola, Morley, Schachter and Smaje, 1965; Beilenson, Schachter and Smaje, 1968; Schachter, 1969; 1970; Karpinski, Barton, and Schachter, 1971; Schachter, Barton and Karpinski, 1973; Schachter, Barton, Uddin, Karpinski and Sanders, 1977) who claim that secretion and functional hyperaemia still occur without involvement of the kallikrein-kinin system. Some evidence for and against the kallikrein-kinin system in functional vasodilatation are described below.

The evidence for the existence of vasomotor nerves (constrictor and dilator) goes back to Claude Bernard (1858) who had observed that vasodilatation followed stimulation of the parasympathetic nerve while vasoconstriction resulted from stimulation of the sympathetic. The assumption was made that autonomic nerves fibers were ending close to and acting directly on the blood vessels. Heidenhain (1872) observed, as Ludwig had done before (1851), that stimulation of the chorda tympani nerve in the cat resulted in secretion of saliva from the submandibular gland but he noticed also that atropine blocked the secretion without affecting the vasodilatation. Early in this century, Barcroft and co-workers (Barcroft and Müller, 1912; Barcroft and Piper, 1912; Barcroft, 1914) concluded from their work that functional vasodilatation was the result of the secretory metabolic activity which was not affected by atropine whereas the secretion was. However, Bayliss (1923) maintained that there was no relationship between the increased metabolic rate and the increased vasodilatation produced by the chorda tympani nerve stimulation after administration of atropine.

If vasodilatation was going to be induced by a chemical mediator as suggested by those who supported the theory of vasodilatation linked to metabolism, no such mediator was then known. However, in 1936, Ungar and Parrot postulated that kallikrein, which had just been discovered in the submandibular gland (Werle and von Roden, 1936), was responsible for the vasodilatation in the submandibular gland following stimulation of the chorda tympani nerve.

Twenty years later, Hilton and Lewis published a series of paper (Hilton 1960, 1962, 1963; Hilton and Lewis, 1955 a,b, 1956, 1958; Lewis, 1960, 1962, 1963) in which they restated that vasodilatation was mediated by kallikrein not only in the submandibular gland but was a general mechanism in many organs. Hilton and Lewis concluded that true parasympathetic vasodilator nerve fibers do not exist and that the vasodilatation accompanying parasympathetic nerve stimulation is due to kallikrein released from secretory cells, then passing into the interstitial fluid where it acts on its globulin substrate to release bradykinin.

However, as mentioned earlier, Schachter's group challenged the Hilton and Lewis theory. Their evidence is based on experiments showing that:

a) Duplication of vasodilatation produced by stimulation of the chorda tympani nerve was not possible by injection of saliva into the arterial supply of the gland (Bhoola et al., 1965).

b) Vasodilation following stimulation of the chorda tympani nerve of gland perfused with horse serum (from which kinin cannot be released by cat saliva) was still maximal (Bhoola et al., 1965).

c) Vasodilatation was unaffected in glands almost completely depleted of kallikrein by chronic duct ligation and sympathetic stimulation (Beilenson et al., 1968).

d) Saliva collected after sympathetic nerve stimulation had a much higher concentration of kallikrein than saliva collected after parasympathetic nerve stimulation (Beilenson et al., 1968).

e) The latency between nerve stimulation and vasodilatation was too short (as low as 350 msec) to explain a vasodilatation mechanism based on an enzymatic system such as the kallikrein-kinin system (Karpinski et al, 1971; Schachter et al., 1973).

f) Kallikrein was probably located in secretory granules contained in the striated duct cells of the submandibular gland since these granules were depleted by sympathetic nerve stimulation (Schachter et al., 1977).

Other workers have also presented evidence which challenges the Hilton and Lewis theory. Among those, Snell and Garrett (1958) and Garrett,(1966 a,b) showed that blood vessels of the rat's submandibular gland receive a dual parasympathetic and sympathetic innervation and that branches of the chorda tympani nerve reach the duct and arteries. Terroux, Sekelj and Burgen (1959) demonstrated that in the dog submandibular gland, salivation can be reduced by atropine without affecting the vasodilatation induced by stimulation of the chorda tympani nerve, and suggested that the metabolic and vasodilator effects of chorda tympani nerve stimulation could be separated. Skinner and Webster (1968) suggested that most of the chorda vasodilatation was due to the presence of adrenergic neurones in the chorda tympani nerve, causing vasodilatation via β - adrenergic receptors which are present on the blood vessels. Their evidence was based on experiments showing that chorda vasodilatation was still present in cats pre-treated with kallidin inactivator while vasodilatation which normally could be induced by close-arterial injection of kallidin was abolished in such glands. Frewin, McConnell and Downey (1973) also showed that kallikrein is not a potentiator of vasodilatation in sweat glands.

Finally, bringing the two theories together, Gautvik and co-workers (see Brandtzaeg et al., 1976) postulated that in the cat two different mechanisms regulate functional vasodilatation, one involving vasodilator nerves, and the other, the kallikrein-kinin system.

The question is thus far from being resolved yet and the present results, the localization of kallikrein in some glandular tissue, might help to throw some light on the subject.

1.4.2 Plasma kallikrein-kinin system.

Keele (1957) had observed that human plasma released a kinin-like pain-producing substance (PPS) on contact with glass. Later, Margolis (1958) found that this kinin was not released from blood of patients with Hageman trait, whose blood does not clot in glass. In

1973, Wuepper observed that prekallikrein corrected a coagulation defect in plasma from patient with the so-called Fletcher Factor deficiencey (Hathaway, Belhasen and Hathaway, 1965). Wuepper's discovery came a long time after the closely related observations of Schachter's group saying: "that those individuals deficient in the so-called Hageman Factor (HF) in their plasma may in fact be deficient in the inactive kallikrein precursor." (Bhoola, Calle and Schachter, 1960). Plasma deficient in Fletcher Factor, even if distinct from HF-deficient plasma, fails also to release kinin on contact with glass and similarly no haemostatic problems or coagulation defects are observed in patients with Fletcher Factor deficiency.

Some of the probable relationships between the kallikrein-kinin system in plasma and the blood coagulation system are illustrated in fig. 1, (Han, komiya, Kato, Iwanaga and Suzuki, 1975) . As indicated, kallikrein also activates the pre-Hageman Factor and provides a positive feedback system to maintain HF formation (Cochrane, Revak, Aikin and Wuepper, 1970; Cochrane, Revak and Wuepper, 1973 a,b).

By activating pre-HF, kallikrein is believed to correct other deficiencies present in Fletcher plasma, such as defects in fibrinolysis, kinin-generation and chemotaxis (Weiss, Gallin and Kaplan, 1974). It thus appears that some of the physiological rôles of kallikreins like activation of pre-HF, are not due to the kinin they release. A negative feedback mechanism, implicating HRP or Histidine-Rich-Peptide released from kininogen by plasma kallikrein (Han et al., 1975; Han, Komiya, Kato, Iwanaga and Suzuki, 1975) has been proposed.
HRP in contrast to kallikrein, inhibits the activation of pre-HF (see

Fig. 1. Relationship Between Plasma Kallikrein and Blood Coagulating Systems.

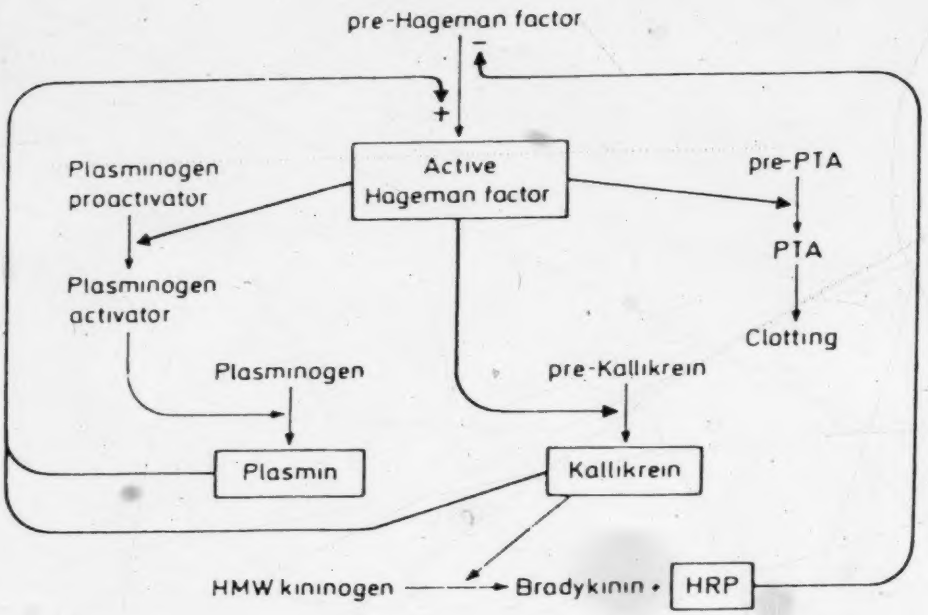

pre-Hageman factor
+
−
Active Hageman factor
Plasminogen proactivator
Plasminogen activator
Plasminogen
Plasmin
pre-PTA
PTA
Clotting
pre-Kallikrein
Kallikrein
HMW kininogen
Bradykinin +
HRP

fig. 1).

1.4.3 Renal kallikrein and hypertension.

Kallikrein was originally discovered in urine (Frey, 1926), but was not reported in kidney until thirty years later (Werle, 1960; Werle and Vögel, 1960). The localization of renal kallikrein in the distal tubular cells was achieved only recently (Orstavik et al., 1976). The same researchers have also demonstrated that kallikrein is synthesized in the kidney (Nustad, Vaaje and Pierce, 1975) and that urinary kallikreins are similar in their biological activity and immunological properties to those in the kidney (Nustad and Pierce, 1974). Injection of kinins into the renal artery produces vasodilatation, increased blood flow and natriuresis (Jacobson, 1970). According to some investigators, there is a correlation between urinary kallikrein and sodium reabsorption (Marin-Grez, Cottone and Carretero, 1972; Adetuyibi and Mills, 1972) whereas others deny it (Croxatto and San Martin, 1970; Geller, Margolius, Pisano and Keiser, 1972; Margolius, Horwitz, Pisano and Keiser, 1974 a; Margolius, Horwitz, Geller, Alexander, Gill Pisano and Keiser, 1974 b).

Urinary kallikrein excretion has been found to be subnormal in hypertensive patients (Margolius et al., 1974 a,b; Margolius, Horwitz, Pisano and Keiser, 1976) and animals (Croxatto and San Martin, 1970; Keiser, Geller, Margolius and Pisano, 1976) but increased in primary aldosteronism (Margolius et al., 1974 a). Thus, aldosterone appears to regulate kallikrein secretion and this secretion increases after a low sodium or high potassium intake (Margolius et al., 1976).

The actions of kinins in the kidney appears to be correlated to the actions of prostaglandins resulting in the facilitation of salt and water excretion (McGiff, Itskovitz, Terragno and Wong, 1976; Nasjletti and Colina-Chourio, 1976).

A further indication that the kallikrein-kinin system is involved in electrolyte and water balance in the kidney was concluded by selective inhibition of bradykinin by its antibody, resulting in cessation of natriuresis induced by infusion of isotonic saline (Marin-Grez, 1974).

1.4.4 Kallikrein and reproduction.

It had been observed that one or many of the components of the kallikrein-kinin system vary in concentration during the oestrous and menstrual cycles, pregnancy and parturition (Werle, 1956; 1960; McCormick and Senior, 1974; McDonald and Perks, 1976; Senior and Whalley, 1976). Because of these cyclic variations, it has been suggested that the kallikrein-kinin system may have an effect on ovulation and parturition. Moreover, it has been shown that kinins have, contrary to their general vasodilator effect, a vasoconstrictor action on the umbilical vein and artery, suggesting that kallikrein is activated at birth and is playing a rôle in the conversion from pre to postnatal circulation (Melmon, Cline, Hughes and Nies, 1968).

Schachter and co-workers reported the presence of kallikrein in the prostate and coagulating gland of the guinea-pig (Bhoola et al., 1962). Its localization was part of this investigation (see results).

It had been suggested that kinins induce sperm motility and sperm count (Schill, 1974, 1975; Ishigami and Kamidano, 1975) as well as facilitating the penetration of the spermatozoa through the zona pellucida of the ovum (Fritz, 1975 a,b).

Further investigation is however necessary to clarify the rôle of the kallikrein-kinin system in reproduction.

1.4.5 Kallikrein and cell proliferation.

It has been reported that kinins stimulate mitosis and DNA synthesis in the thymus and bone marrow of rats injected with hog pancreatic kallikrein (Perris and Whitfield, 1969; Rixon, Whitfield and Bayliss, 1971; Rixon and Whitfield, 1973). It has also been reported that long term treatment with kallikrein results in regeneration of bone marrow in X-irradiated animals and better recovery from radiodermatitis produced by local irradiation (Mandel, Rodesch and Mantz, 1973). The importance of the kallikrein-kinin system in cell proliferation appears to be real but further investigations are needed to clarify these interesting reports.

1.4.6 Modulators and mediators of the kallikrein-kinin system.

Correlation between prostaglandins and the kallikrein-kinin system was demonstrated when kinins were shown to activate synthesis of prostaglandins in the kidney (Terragno, Lonigro, Malik and McGiff, 1972; McGiff et al., 1976; Colina-Chourio, McGiff, Miller and Nasjletti, 1976). Infusion of kinins or kallikrein into the renal artery resulted in increased concentrations of prostaglandins in urine.Bradykinin also releases prostaglandins from isolated guinea-pig lung (Palmer et al., 1973) and dog spleen (Ferreira, Moncado and Vane, 1973).

Kinins also appear to increase cyclic A M P production through prostaglandin formation, since it is blocked by aspirin like drugs which are inhibitors of prostaglandin synthesis (Stoner, Mangoniello and Vaughan, 1973). Recently, Bhoola and co-workers, from studies of cyclic nucleotides in slices of submandibular gland, suggested that both cyclic A M P and cyclic G M P and also calcium are mediators of the kallikrein secretion (Albano, Bhoola, Heap and Lemon, 1976). A relationship between cyclic nucleotides, prostaglandins and the kallikrein-kinin system has been demonstrated but is far from being well defined.

1.4.7 Kallikrein-kinin system in membrane transport.

The implication of kallikrein transport of electrolytes, amino acids and proteins across membranes has not been clearly demonstrated but there appears to be correlation for example, between secretion of potassium and kallikrein concentration. The distal tubular cells of the kidney like the duct system in the salivary glands are the site of kallikrein in these organs and are also responsible for the secretion of potassium (Schneyer, Young and Schneyer, 1972). Moreover, aldosterone which regulates potassium secretion, has a pronounced effect on kallikrein release (Young, McCaa, Pan and Guyton, 1976), giving further evidence for the implication of the kallikrein-kinin system in transport. However, these effects might be only secondary to other kinins actions, like vasodilatation.

1.4.8 General considerations.

As discussed above, due to the various effects of kallikreins and kinins on e.g., cell proliferation, membrane transport, and blood coagulation, it appears that the kallikrein-kinin system has not a unique and specific function. The diversity of actions might be explained by the fact that kininogenases are part of the larger group of serine proteases, which have evolved from a common ancestral origin to various closely related forms participating in a wide array of biological activities (Stroud, 1974).

The mechanism of kallikrein action was generally considered to be due to the kinins which they released, but there are exceptions, like direct activation of pre-Hageman Factor by the kallikrein molecule itself. Therefore, as Schachter wrote, "It seems that the biological significance of the kinins and of the endogenous agents which release them is far from clear. Their major significance may well lie in a biological system, which for the present, is not appearent to us." (Schachter, 1968). This 10 year old statement is still as valid today as it was then.

2. Submandibular gland kallikrein.

2.1 Historical survey.

As stated earlier, Werle and von Roden were the first to associate a vasodepressor substance found in saliva with kallikrein (Werle and von Roden, 1936; 1939). Other researchers had observed that an acetylcholine-like substance was present in perfusates from cat and dog submandibular glands but could not identify it (Gibbs and Szeloczey, 1932; Henderson and Roepke, 1933). Others also reported the presence of a vasodilator substance in saliva (Secker 1934 a,b; Gibbs, 1935; Larson, 1935) and confirmed that the vascular effects were not due to acetylcholine or to adrenaline. There was some indication that this substance was a large, high molecular weight molecule (Larson, 1935) but even further studies by Feldberg and Guimarais (1935) failed to identify the vasodepressor substance, concluding only that is was not acetylcholine, choline, histamine or any other known depressor substance. The latter authors noted however that there was greater depressor activity in sympathetic than parasympathetic saliva and that the depressor activity decreased in successive saliva samples.

2.2 General properties.

Among the salivary glands, the highest concentration of kallikrein is found in the submandibular gland, as reported originally by Werle and von Roden (1936). Only small concentrations have been reported in parotid and sublingual glands. Salivary kallikrein has been isolated from saliva (Moriya, Moriwaki, Yamazaki, Akimoto and Fukushima, 1966; Fujimoto, Moriya and Moriwaki; 1973) and from submandibular glands (Moriwaki et al., 1976; Lemon, Förg-Brey and Fritz, 1976; Brandtzaeg, Gautvik, Nustad and Pierce, 1976; Porcelli, Cozzari, DiIorio, Croxatto and Angeletti, 1976).

From early investigations, based on effects of denervation and correlation with kallikrein content, it was believed that kallikrein was present in demi-lune cells (Emmelin and Henriksson, 1953). Bhoola and co-workers reported that the acinar cells were the likely site of kallikrein storage (Bhoola & Heap, 1970). Howeyer recent investigations contradict these early findings, demonstrating that kallikrein is found exclusively in the duct system of the submandibular gland in the rat (Brandtzaeg et al., 1976; Orstavik, Brandtzaeg, Nustad and Halvorsen, 1975), the pig (Dietl et al., 1978), the cat (Hojima, Maranda, Moriwaki and Schachter, 1977) and the guinea-pig (Scnachter, Maranda and Moriwaki 1978). The localization of kallikrein is further discussed in section 3 of this introduction.

2.3 Factors affecting the concentration of kallikrein in submandibular gland and saliva.

2.3.1 Age and diet.

Submandibular kallikrein seems to appear early after birth, being absent in submandibular glands from foetal and neonatal man and animals. In human saliva, kallikrein concentration is up to five times higher in older people (45 to 78) than in teenagers (Sallay and Nador, 1950). Diet does not appear to influence the kallikrein concentration of submandibular gland contrary to the case with plasma, urinary and pancreatic kallikrein.

2.3.2 Denervation.

Reduction of depressor activity in saliva produced by injection of adrenaline was observed in cats which had their chorda tympani nerve severed (Emmelin and Henriksson, 1953). Depressor activity was also reduced in submandibular gland extracts prepared two to four weeks after chorda denervation. However, the depressor activity of saliva was not markedly affected in cat which had their superior cervical ganglion removed.

2.3.3 Duct ligation.

After chronic duct ligation, the kallikrein concentration in submandibular gland extracts of rabbits (Mattioli and Mattioli, 1947, a,b) rats, mice and dog (Werle, Vogel and Lentrodt, 1960; Werle and Trautschold, 1963) was greatly reduced compared to contralateral unligated glands in which kallikrein concentration was slightly increased. The same was reported in the cat submandibular gland (Beilenson et al., 1968).

2.3.4 Nerve stimulation.

Beilenson et al., 1968, were the first to demonstrate the effect of parasympathetic and sympathetic nerve stimulation on the kallikrein content of the submaxillary gland and saliva. Glandular kallikrein was almost completely depleted after sympathetic stimulation, the reduction ranging from 87% after three minutes actual stimulation time to 99.4% after 47 minutes. No significant difference could be observed between chorda stimulated submandibular gland and the contralateral unstimulated one.

2.3.5 Duct ligation and sympathetic stimulation.

Duct ligation alone, as reported by Beilenson (1967; Beilenson et al, 1968) decreased total kallikrein concentration in submandibular gland from 116,000 K.U. to 170 K.U. for control and ligated gland respectively. Correspondingly, kallikrein concentration in sympathetic saliva collected from control glands and glands ligated for three days decreased from 470,000 K.U./ml to 150 K.U./ml respectively. Again, sympathetic stimulation was far more effective in reducing kallikrein in the gland than parasympathetic stimulation which was practically ineffective.

2.4 Evidence for the localization of submandibular kallikrein.

Based on various types of investigations such as histochemical staining, ultracentrifugation analysis correlated to electron microscope studies, and finally immunofluorescence staining, localization of kallikrein in submandibular gland was achieved (see next section and results).

3. Localization of kallikrein in submandibular gland.

3.1 Indirect evidence.

One of the first researches that could have helped to localize kallikrein in the submandibular gland was that of Rawlinson (1935) who studied changes in striated ducts after parasympathetic and sympathetic stimulation. Due to the limited techniques available at the time, the only thing he could conclude was that both nerves had effects on the striated ducts without much further refinement.

Almost 20 years later, Emmelin and Henriksson (1953) observed a large reduction in the kallikrein content of submandibular gland extracts prepared two to four weeks after parasympathetic denervation. They had also noted that this fall in depressor activity corresponded well with a reduction in the size of the demi-lune cells of the gland. They did not observe any structural changes in the gland after sympathetic denervation. It was Bhoola and co-workers who first claimed that kallikrein might be stored intracellularly in granules of the guinea-pig submandibular gland (Bhoola and Ogle, 1966). His group and other workers, using various techniques as outlined below, found evidence for the localization of kallikrein in glandular tissue, mostly in studies on the submandibular gland. However, as it will appear later, the results were not always corroborating each other.

3.1.1 Density gradient centrifugation analysis correlated to ultrastructural studies.

Studying cell fractions collected after density gradient centrifugation of submandibular extracts, Bhoola (1968) concluded that kallikrein was absent from mitochondria and lysosomes. His conclusion was based on the fact that no kallikrein activity was detected in fractions having succinic dehydrogenase activity, an enzyme associated with mitochondria, or with β - glucuronidase or acid phosphatase, which are lysosomal enzymes. Later, Bhoola and Heap (1970), using the electron microscope to study populations of granules isolated by ultracentrifugation and comparing them by their appearance and size to the ones observed *in situ*, came to the conclusion that the granules containing the highest kallikrein concentration resembled closely those granules observed in serous acinar cells. It was thus suggested that kallikrein was stored in acinar cells of the submandibular gland of the guinea-pig. This conclusion was further extended to the cat, dog and rabbit, since a similar distribution pattern for kallikrein had been obtained in submandibular glands of these three mammals (Bhoola, 1969).

3.1.2 Histochemical (Periodic Acid Shiff (PAS) and Tryptophan) staining.

Trautschold, Fritz and Werle (1966) had reported that kallikrein was a glycoprotein; it would thus likely be stained with PAS solution thought to react with glycoproteins and polysaccharides. Bhoola and Heap (1969) observed that granules, isolated by density-gradient centrifugation and known to contain kallikrein reacted positively with PAS reagent. They thus extended their investigations by specifically staining sections of submandibular gland with PAS solution. They observed that non-granular PAS material was present in striated duct cells and that granules in serous-acinar cells and in the apical region of intercalated duct cells were also PAS positive. However, PAS reactivity of material in striated ducts was lost after treatment with α - amylase whereas granules from acinar and intercalated duct cells retained their reactivity. This indicated that PAS staining material in striated duct was probably a polysaccharide, like glycogen, and not kallikrein. They concluded that this was further evidence for the storage of kallikrein in acinar cells granules. Intercalated duct cell granules, they concluded, were too small to be considered, sedimenting with mitochondria.

More recently, Garrett and Kidd (1975) found that the apical portion of striated duct cells in the submandibular gland of the cat contained material specific for PAS but which was also diastase-resistant, indicating that it was not glycogen, which is diastase-labile. They associated that material with neutral mucin. Harrison and co-workers (Harrison and Garrett, 1975) had already observed that neutral mucin was depleted, like kallikrein, after submandibular duct ligation, strongly suggesting that this neutral mucin may represent secretory material containing kallikrein. Moreover, Garrett and Kidd (1975) noted that neutral mucin was depleted, like kallikrein, after sympathetic stimulation, as reported by Beilenson et al., (1968), whereas no effect was produced by parasympathetic stimulaation (see next section). This reinforced their hypothesis that kallikrein was contained in secretory material corresponding to neutral mucin. Since tryptophan had been found to be a component of pancreatic (Moriya, 1959; Fritz, Eckert and Werle, 1967) and salivary kallikrein (Frey, Kraut and Werle, 1968; Fujimoto et al., 1973), these authors (Garrett and Kidd, 1975) specifically stained sections for tryptophan and observed that tryptophan-specific material was also located at the apical portion of striated duct cells. Moreover, this was also depleted after sympathetic stimulation, being a further indication that kallikrein is present in the apical secretory material.

3.1.3 Correlation of kallikrein depletion with ultrastructural studies.

As mentioned above, Beilenson et al., (1968) reported that kallikrein content of submandibular gland extracts of the cat was reduced considerably if not completely abolished after duct ligation and/or sympathetic stimulation. Recently, electron microscope studies of submandibular gland depleted of kallikrein (Barton, Sanders, Schachter and Uddin, 1975; Schachter et al., 1977) indicated that kallikrein was likely located in secretory granules of striated duct cells, since disappearance of those granules corresponded with the kallikrein depletion in those glands while no change was observed in acinar cells after sympathetic stimuation. Moreover, parasympathetic stimulation resulted in the disappearance of secretory granules from the acinar cells but there was no reduction in kallikrein concentration. There was no clear indication about demi-lune cells, the results not being consistent. Essentially the same observations were made by Garrett and Kidd (1975).

More recently, Uddin and Tyler (1978) observed that both parasympathetic and sympathetic stimulation resulted in depletion of secretory granules from the demi-lune cells in the submandibular gland, suggesting again that demi-lune cells were an unlikely source kallikrein.

As seen from the above investigations, there was still some uncertainty concerning the localization of kallikrein in the submandibular gland, even if there was fairly good evidence suggesting that kallikrein was present in the secretory material in the apical region of the striated duct cells. Direct evidence, by immunohistochemical techniques, was necessary to definitely answer the question.

3.2 Direct evidence.

Antibodies, specific for particular substances, and tagged with markers like rhodamine and fluorescein have been used for many years to locate various biologically active compounds. This immunofluorescence technique requires the purification of the compound to be located, its injection as antigen in animals to induce antibody formation and collection of the blood from the immunized animals. The antibody present in the immune serum can then be purified and tagged with a marker and used directly to stain sections of the tissue in which the antigen is believed to be present. This is the direct immunofluorescence technique. An indirect technique using two antibodies, the first one specific for the antigen and the second-one specific for the first antibody; in this case, the first antibody is the antigen for the second antibody. The second antibody can be bought commercially and the first antibody does not have to be purified, crude immuneserum being used as a source of antibody.

Both direct and indirect immunofluorescence staining have been used for the localization of kallikrein in glandular tissue. The Norwegians (Orstavik, Brandtzaeg and Nustad, 1975) were the first to prepare antibody to kallikrein and to use it for the localization of kallikrein, as described below.

3.2.1 Immunocytochemistry: Rat submandibular kallikrein.

The first successful attempt to definitely localize kallikrein in glandular tissue was reported only three years ago. After purification of rat urinary kallikrein and preparation of its antibody, Orstavik and co-workers (Orstavik, Brandtzaeg and Nustad, 1975) noted that there was cross-antigenicity between urinary kallikrein and salivary gland kallikrein. They thus used the antibody specific for urinary kallikrein to localize by direct immunofluorescence the kallikrein in submandibular and sublingual glands of the rat. They found that most of the kallikrein appeared to be in cytoplasmic granules of granular tubules and some also in the striated duct cells of the submandibular gland. In sublingual gland, kallikrein was found in striated duct cells and also as a luminal rim in main duct cells. Acini were negative for kallikrein in both glands. Repeating their experiments, the same group of workers (Orstavik et al., 1975) found essentially the same results except that striated ducts showed a bright luminal rim which was also occasionally seen in excretory ducts of the submandibular gland.

3.2.2 Immunocytochemistry: Kallikrein in kidney and pancreas of the rat.

Using immunofluorescence technique again, it took the same workers some time to resolve some fixation problems with the kidney tissue before being able to successfully localize kallikrein in that organ (Orstavik et al, 1976). Renal kallikrein was found in the distal tubular cells, a structure resembling the striated ducts of the submandbular gland. Moreover, using antibody specific for rat submandibular kallikrein and the direct immunofluorescence technique, Proud et al., (1977) demonstrated that kallikrein was located in the acini of the rat pancreas. Since fluorescence was mainly observed at the apical portion of the acinar cells, these authors suggested, as later did Dietl and co-workers (1978) for porcine pancreatic kallikrein, that kallikrein is located, like other proteinases, in the acinar zymogen granules.

3.2.3 Immunocytochemistry: Hog pancreas and submandibular kallikrein.

Having demonstrated the immunological cross-reactivity between porcine glandular kallikreins from pancreas, submandibular gland and urine (Fritz, Fiedler, Dietl, Warwa, Truscheit, Kolb, Mair and Tschesche, 1977), Dietl and co-workers (1978) used the indirect immunofluorescence technique to localize kallikrein in pancreas, submandibular gland and kidney with antibodies directed against each of these kallikreins. Kallikrein was found in the acinar cells of the pancreas and at the apical portion of striated and collecting duct cells of the submandibular gland. The same immunofluorescence pattern resulted from the use of anti-pancreatic, anti-submandibular or anti-urinary kallikrein antibody, showing again that there was cross-reactivity between these antibodies. However, the same technique was unsuccessful with kidney tissue, the authors suggesting that it might be due to the low amount of kallikrein present in that tissue or to the fact that kallikrein might bind to kidney organelles in such a way that its antigenic sites were buried.

3.3 General considerations.

As described, considerable progress had been made in the direct localization of kallikrein in glandular tissue. Immunofluorescence technique was also employed in investigations described in this thesis, resulting in the localization of kallikrein in guinea-pig coagulating and submandibular glands and in the submandibular and the parotid glands of the cat. It is hoped that the localization of kallikrein in tissue help to further define its physiological significance.

MATERIALS AND METHODS

1. Source, purification and activities of the various kininogenases utilized.

The purified kallikreins used in the experiments described in this work were generously provided by Dr. C. Moriwaki from the Faculty of Pharmaceutical Sciences, Science University of Tokyo, Japan.

As described in the corresponding references, the guinea-pig coagulating gland kallikrein (CGK) (Moriwaki, Nuki, Fujimoto and Moriya, 1974), the cat submandibular gland kallikrein (CSK) (Moriwaki, Hojima and Schachter, 1976) and the dog renal kallikrein (DRK) (Moriwaki, Miyazaki, Matsuda, Moriya, Fujimoto and Veki, 1976) were purified by a series of standard protein purification techniques such as acetone fractionation, dialysis, DEAE-cellulose chromatography, Sephadex gel filtration and Ampholine isoelectric fractionation.

The respective activities of the purified kallikreins and their molecular weights are indicated in Fig. 2. All purified kallikreins had a high kininogenase activity as assayed by both chemical (esterolytic) and bio-assay tests. They all resulted in single band on disk electrophoresis.

2. Antibodies to purified kallikreins.

2.1 Induction of antibody formation in rabbits.

Antibody formation was induced in rabbits (New Zealand white) by subcutaneous injections of the purified kallikreins in solution in

Fig. 2. Esterolytic and Vasodilator Activities of purified Kallikreins. (C.Moriwaki, personal communication).

	Vasodilator Activity KU/mg	Esterolytic (TAME) Activity μ Moles/min
CGK	420	38.0
CSK	1260	11.6
DRK	66	1.1

Guinea-pig's coagulating gland kallikrein (CGK), cat's submandibular gland kallikrein (CSK) and dog's renal kallikrein (DRK) were purified and their homogeneity verified by Moriwaki and co-workers. Samples were graciously made available to us.
Activities are expressed per mg of purified material.
TAME is p-Tosyl-l-Arginine Methyl Ester HCl.

0.9% NaCl and emulsified with complete Freund's adjuvant for the first injection and with incomplete adjuvant for the following two injections. At each weekly injection, 0.25 mg of purified kallikrein in a total volume of 0.30 ml was injected at the base of the back of the neck at several distinct but adjacent sites so that the solution was injected over an area of approximately 5 sq cm. Antibodies to pure kallikrein were obtained by collecting blood by ear vein puncture 15 days after the third injection of the antigen. The serum was then separated from the whole blood by coagulation at room temperature for 1 hour, clot retraction in the cold (4°C) for 1 hour and centrifugation in the cold at 4000 x g for 15 min. The sera obtained were protected against bacterial contamination by the addition of sodium azide (final concentration of 0.01%) and kept frozen if not immediately used. Control sera were obtained in a similar manner from the same rabbits before immunization with the respective purified kininogenases.

2.2 Purity and specificity of the antibodies.

2.2.1 Immunodiffusion.

The purity and specifity of the antibodies were tested by the immunodiffusion technique (Ouchterlony, 1958). Immunodiffusion plates were prepared using 1% Difco Agar in phosphate buffered saline (PBS), pH 7.2, containing 0.025% sodium azide. 12.5 ml of hot agar solution was poured in Petri dishes (100 mm in diameter), resulting in a layer of agar approximately 3 mm thick. Holes, 4 mm in diameter, were punctured in the agar with a #3 cork borer. The volume of the well was then approximately 40 µl. Various concentrations of antigens and antibodies were used to obtain the optimum precipitation pattern (see Results).

2.2.2 Inhibition studies.

Varying concentrations of antibodies from antisera were incubated for 15 min at room temperature with their respective antigens. The incubated mixture was then tested by immunodiffusion against the appropriate antigen (see Results for details).

Similar incubated mixtures of antigen-antibody were also tested for their esterolytic activity by the spectrophotometric method of Trautschold (1970) using benzoyl-L-arginine ethyl ester (BAEe) as substrate, and for their kininogenase activity by using the biological assay preparation described by Moriwaki and Schachter (1971), which consists of recording the smooth muscle contractile effect of kallikrein on a piece of guinea-pig ileum in an organ bath.

Finally, the specificity of the respective antibody against its antigen was verified by inhibiting the antibody present in immune-serum by its antigen prior to immunofluorescence staining of tissue sections. The procedure consisted in incubating 0.1 - 0.2 ml of immune serum with antigen for 30 min at room temperature. The resulting mixture was thereafter used for immunofluorescence staining in place of neat serum. Antigens were used in concentrations ten times higher than those revealed by maximum precipitation in the immunodiffusion tests. Controls consisted of immune sera incubated with PBS pH 8.0.

2.2.3 Cross-antigenicity.

The cross-antigenicity of the purified kallikreins or of the kallikreins present in various gland extracts was verified by immunodiffusion of the three antibodies available versus various antigens and gland extracts of the same or different species (see Results for details).

The presence or absence of cross-antigenicity was also demonstrated by immunofluorescence staining using immune serum to stain sections of various tissues of the same animal.

3. Animals: Pre-operative, organ dissection and tissue preparation procedures.

3.1 Guinea-pig.

3.1.1 Anaesthesia.

Guinea-pigs of either sex (except for experiments on coagulating and prostate glands, where males were used), weighing 300-500g and starved overnight, were anaesthetized with pentobarbitone sodium (40 mg Kg^{-1} I.P.).

3.1.2 Coagulating and prostate gland dissection.

Male animals were placed in a supine position on the operation table, the skin of the lower abdomen shaved and cleaned. A midline incision was made through the skin and the underlying fascia to get access to the internal organs. The seminal vesicles were identified and carefully pulled out of the abdominal cavity. The coagulating gland and prostate could then be observed at the base of the seminal vesicles. By careful dissection, the coagulating gland, consisting of larger and more transparent lobules than those of the prostate and located in a more ventral and anterior position, could then be separated from the seminal vesicles and the prostate and taken out. The same applies for the prostate gland, which consists of two dorsal lobes and two thin pear-shaped ventral lobes, having a less dense muscular and fibrous framework than the coagulating gland (Walker, 1910; Cooper & Schiller, 1975). The excised tissue was then immediately rinsed in 0.9% NaCl and placed in cold fixative. Other portions of the glands were dissected out, rinsed in saline, weighed and freeze-dried. Aqueous extracts were prepared with these freeze-dried glands, centrifuged at 4000 x g for 15 min, and the supernatant freeze-dried again. The resulting freeze-dried powders could then be used for esterase and kininogenase activity determination.

3.1.3 Guinea-pig sperm.

The sperm was collected as described by Allen (1976). The testes of the anaesthetized animals were dissected out by performing a small incision in the scrotum sac. The vas deferens and epididymis were identified and separated from surrounding tissues. The distal end of the vas deferens was ligatured, cut and cannulated with a short piece of polyethylene tubing. Once the epididymis was separated from the testes, by the use of a small syringe, PBS pH 8.0 was introduced in the vas deferens to reverse wash its contents and the contents of the epididymis. The sperm was collected drop-wise at the proximal end of the epididymis. Sperm smears were prepared, and in some experiments, pieces of epididymis were excised, fixed and embedded for further investigation.

3.1.4 Submandibular gland dissection.

Anaesthetized animals of either sex, preferably lean, were placed in a supine position on the operation table. The skin of the ventral portion of the neck was shaved and cleaned and a midline incision was started 1 cm from the mandible and extended posteriorly for 3 - 4 cm. The submandibular glands can be located on either side of the incision, just underlying the skin. To identify the glands with certainty, the main excretory duct can be identified, dissected out and followed to the gland.

Submandibular gland fragments were then excised with the blood supply still intact, rinsed in 0.9% NaCl and immediately placed in cold fixative. The remaining of the gland was then removed, rinsed in saline, weighed and freeze-dried after mincing the tissue with scissors or scalpel. Esterase and kininogenase activities of the gland can be determined using the freeze-dried portion of the gland.

3.1.5 Castration experiments.

Animals were anaesthetized, attached to the operating board and the hair of the scrotum shaved and cleaned. A small incision (1 - 1.5 cm long) was made on each side, over the location of the testes, and each testis was pressed out of the scrotum sac and dissected out from the surrounding tissue. The blood vessels and vas deferens were tied with double nylon ligature, the testes excised and the incision closed with stainless steel clips.

On the day of castration, a control guinea-pig had its right submandibular gland removed, pieces taken out for immunofluorescence staining and the rest of the gland freeze-dried for further investigation. Two other guinea-pigs were castrated on the same day and their right submandibular gland also excised, fragments of it processed for immunofluorescence staining and the remaining freeze-dried. Three weeks after castration, the left submandibular and coagulating glands of these animals were dissected out, samples taken out for immunofluorescence staining, and the rest of the gland freeze-dried for esterase activity determination.

3.2 Cat.

3.2.1 Anaesthesia.

For acute experiments, cats of either sex weighing 2 - 4 Kg and starved overnight, were anaesthetized with chloralose (80 mg Kg^{-1}) injected intravenously by the femoral vein, which had been cannulated. Anaesthesia was first induced by chloroform in an air-tight chamber. For chronic experiments, such as submandibular duct ligation, cats were anaesthetized with pentobarbitone sodium (30 mg Kg^{-1} I.P.) under aseptic conditions.

3.2.2 Salivary glands dissection.

Anaesthetized animals were placed in a supine position on the operation table. The skin of the ventral portion of the neck was shaved and cleaned. A midline incision was made from 2 cm away from the mandible and extended caudally for 5 - 6 cm. The skin was then carefully dissected from the underlying tissue. The submandibular glands can be localized on either side of the trachea, just under the skin, in a position corresponding to the junction of the transverse vein to the external jugular vein. The sublingual glands are next to the submandibular glands, but form a distinct gland with separate blood supply and secretory duct. They are located in a more anterior and slightly more ventral position than the submandibular glands (Crouch, 1969). The parotid glands, having a more pinkish appearance than either the submandibular or sublingual glands, are on either side of the trachea in a position corresponding to the junction of the transverse vein to the external jugular but in a more dorsal position than the submandibular glands. For positive identification of the glands, the main excretory duct of each gland can be identified, dissected out and followed to each particular gland.

Samples of the gland were removed whilst the blood flow was still intact, rinsed in 0.9% NaCl and prepared for light, fluorescence and/or electron microscopy. The rest of the glands were then excised, rinsed in saline, weighed, minced with scissors and freeze-dried. Esterase and kininogenase activities of the glands could then be determined using these freeze-dried glands.

3.2.3 Submandibular gland main excretory duct.

After submandibular gland dissection, its excretory duct (Wharton's) was cleared of surrounding tissues and dissected out from the hilus of the gland for a three cm length. A portion of the duct was excised with the blood circulation still intact, cut in short pieces, rinsed in 0.9% NaCl and fixed immediately in 95% ethanol before being processed through embedding, sectioning and immunofluorescence staining.

3.2.4 Kidney and pancreas dissection.

Anaesthetized cats, starved overnight, were placed in a supine position on the operation table. The skin of the abdomen was shaved and cleaned and a midline incision made through the skin and the underlying fascia along the linea alba. The intestines were partly pulled out or pushed sideways to reach the location of the kidney or the pancreas. The organs were identified, dissected clear of surrounding tissue and fragments excised with the blood supply still intact. Some of the tissue excised was rinsed and fixed immediately for microscopical examination. Other fragments were rinsed, weighed, minced and freeze-dried.

3.3 Dog.

3.3.1 Anaesthesia.

Dogs were anaesthetized with sodium pentobarbitone (30 mg Kg^{-1} I.V.), the anaesthetic being introduced through a cannula inserted into the femoral vein.

3.3.2 Kidney dissection.

Anaesthetized dogs were placed in a supine position on the operation table. The skin of the abdomen was shaved and cleaned and a midline incision made along the linea alba through the skin and the underlying fascia. The intestines were partially pulled out or pushed aside to reach the kidneys. Small fragments of the gland were excised with the blood supply still intact, rinsed in 0.9% NaCl and fixed immediately for histological and immunofluorescence studies.

4. Ligation and stimulation experiments on submandibular glands.

4.1 Ligation experiments.

These experiments were done in both the cat and the guinea-pig.

The right submandibular main excretory duct was exposed under aseptic conditions and a double nylon ligature was tied around it. Acute experiments, such as nerve stimulation and excision of tissue, were performed 4 - 7 days after ligation of the ducts. The contralateral gland was used as a control in all experiments.

4.2 Stimulation experiments.

These experiments were performed only in the cat. The cervical sympathetic and chorda lingual nerves were dissected, cut and their distal ends mounted on bipolar platinum electrodes. The cervical sympathetic nerve was dissected from the vagus prior to stimulation. Nerves and electrodes were kept immersed in a pool of warm liquid paraffin. The sympathetic nerve was stimulated at 20 Hz for 10 sec followed by intervals of 20 sec rest; the total time of actual nerve stimulation varied from 10 to 30 min in different experiments. The parasympathetic chorda lingual nerve was stimulated at 10 Hz for 10 sec followed by 20 sec rest for continuous intervals of 5 min followed by 5 min rest, with a total stimulation time of 20 min. The stimulation consisted of square wave pulses, duration of 0.5 msec, voltage of 7 - 10 V. The collection of saliva was done by exposing the submandibular duct and cannulating it with a fine glass or polyethylene cannula. The saliva was then weighed and frozen until further analysis, like esterase and kininogenase activity determination.

5. Immunofluorescence.

5.1 Tissue fixation.

Various fixation techniques were tried to maintain good structural appearance of the tissue under microscopy and to retain the antigenic activity of the kininogenases present in the tissues.

Most of the initial experimentation was carried out on guinea-pig coagulating glands, except as noted.

5.1.1 Freeze-drying.

Coagulating gland fragments, 4 - 8 mm^3, were frozen by immersion in a mixture of dry ice and acetone, and freeze-died overnight. The resulting coagulating gland pieces were embedded under vacuum in wax (Paraplast, Scientific Products Co.) at 56°C. Sectioning and staining were carried out (as described later) to verify the structural appearance of the tissue.

5.1.2 Frozen sections.

Samples of guinea-pig coagulating gland were excised with the blood flow still intact, rinsed in cold 0.9% NaCl and frozen in isopentane at -20°C in a mixture of dry ice and acetone. Sections 6 - 25 μm thick were cut in cryostat (Lipshaw freezing microtome), collected on glass cover slips and stained by the haematoxylin-eosin technique for microscopic examination.

5.1.3 Fixatives.

Various fixatives, including 95% methyl alcohol, 95% ethyl alcohol, 100% acetone, 5% formaldehyde (freshly prepared from paraformaldehyde) in 95% ethyl alcohol, were tried on guinea-pig coagulating gland fragments. The fixation times varied from 2 hours at room temperature to 24 hours at 4°C.

The fixed tissues were then embedded, sectioned, stained and observed under the microscope. For the sections revealing good structural detail, an extract of the corresponding fixed tissue was prepared by grinding pieces of fixed tissue in a Polytron homogenizer equipped with a 1 cm stainless steel head. A mixture ratio of 20 - 30 mg dry tissue per ml of PBS, pH 7.6, was used. The resulting solutions were centrifuged at 4000 x g for 15 min and the supernatant frozen or kept at 4°C. The antigenic activity of the extracts was verified by reacting the fixed tissue extract versus immune-serum (containing coagulating gland kallikrein antibody) by an immunodiffusion test. Fresh gland extract was used as a control. Precipitin arcs would demonstrate the conservation of the antigenic activity throughout fixation. The same procedure was repeated on ethanol fixed and paraffin embedded tissue. Tissue blocks ($8mm^3$) were cleared of paraffin in xylene (3 x 15 min), absolute ethanol (2 x 15 min), 95% ethanol (2 x 15 min), 50% ethanol (2 x 15 min) and PBS pH 7.5 (2 x 30 min). Extracts were prepared and their antigenic activity verified as outlined above.

5.2 Tissue embedding.

After fixation, the tissue blocks were cleared by three successive 1 hour immersions in cold xylene and embedded in paraffin (Paraplast or Paraplast-Plus) at 56°C under vacuum (Tissue Tek II - Vacuum Infiltrator-Lab Tek Products). Stainless-steel embedding dishes (LAB-TEK, 1 cm^2) were used to contain the tissue blocks during the embedding period. The embedded tissue was left to solidify at room temperature and then placed at -20°C for 15 min to help separate the wax blocks from the supporting dishes.

5.3 Sectioning.

Tissue sections, 5 - 10 μm thick, were cut manually using a Leitz 1212 microtome. Long ribbons, containing 30 - 40 serial sections, were separated in portions of two or four sections with a razor blade. These sections were floated on water at 45°C to flatten the sections, and collected on albuminized slides (Fisher Albumin Fixative). The slides were drained and the sections dried on a warm plate at 40°C. Prior to staining, deparaffinization was carried out by immersion of the slides in three successive baths of xylene for 1 min each, followed by three baths of 95% ethanol for 1 min each and then three baths of PBS at pH 8.0, also for 1 min each. For immunofluorescence staining, the tissue was stained immediately after deparaffinization to avoid drying of the tissue.

5.4 Staining.

Fluorescent staining by the indirect method was based on Coon's sandwich technique (Coons, 1958).

The general procedure described by Kawamura (1969) was employed. The deparaffinized tissue sections were incubated at 37°C for 30 min with either undiluted or 1:1 PBS-diluted immune or non-immune (control) sera in a moist chamber. The slides were then rinsed in three successive baths of PBS for 5 min each, counterstained for 2 mins with Evans blue (.005% w/v in PBS) and washed for 5 min in PBS. The sections were then incubated at 37°C for 30 min with FITC (fluorescein isothiocyanate)-labelled rabbit anti-IgG (Miles-Yeda, Israel) in a moist chamber. This labelled anti-IgG had previously been diluted to one-tenth of its original concentration (1% IgG solution with O D ratio at 280/495 nm = 1.7). The slides were then washed in three consecutive baths of PBS (5 min each), dried at room temperature and mounted with fluormount (Edward GURR, London).

A typical slide had four serial sections on it, placed side by side. The deparaffinized tissue sections, still moistened with PBS, had been delineated on the slide with wax pencil (blue eye liner by Revlon was found to be the most satisfactory) to prevent spread and mixing of the added solutions. The first two sections had been treated with non-immune and the last two with immune serum. One section from each pair had then been stained with FITC-labelled rabbit anti-IgG while the other had been covered with PBS. The slide thus had one section showing specific immunofluorescence with three others as control.

Initially, the incubation with the sera and the FITC-labelled rabbit IgG was done in the cold (4°C) over a period of 18 to 24 hours. However, it had been found that the solutions had a tendency to run off the slides and the sections to dry out. The above mentioned procedure (at 37°C for 30 min) proved to be more convenient and as satisfactory.

5.5 Microscope observation.

A Zeiss photomicroscope II with HBO high-pressure mercury lamp was used for fluorescence microscopy. An FITC filter, having a maximum light transmission between 400 and 500 nm, was used as an exciter filter while a Zeiss #53 Barrier filter, blocking transmission of light above 530 nm, was employed. Dark field condenser and objectives were used to improve the contrast (Kawamura, 1969). Zeiss fluorescence free immersion oil was used between the condenser, the slide and the immersion objectives.

5.6 Photography and printing.

Photographs were taken with the 35 mm Zeiss camera incorporated in the microscope. The exposure time, automatically controlled by the Zeiss integrated exposure meter, varied between 10 sec to 1 min, depending on the magnification and the brightness of the section. Spot reading was used in most instances to get proper exposure time. Anscochrome 200 ASA was found to be the most appropriate film, having relatively fine grain for good resolution and high speed for short exposure. Exposure time was kept as short as possible since the fluorescence in the tissue had a tendency to fade out under prolonged illumination. The stained sections were always kept in the dark except during microscopic observation.

Since commercial printing was found to give unsatisfactory results, the color balance of the prints obtained not reflecting the picture observed under the microscope, Cibachrome printing, a process for printing directly from color slides, was employed. The results proved to be very satisfactory, being able to reproduce accurately the appearance of the tissue as seen under fluorescence microscopy. Initially, a Leitz Valoy II enlarger and Cibachrome color filters were used. Later, a Super Chromega D Dichroic II enlarger with color head was employed for printing. The printing paper and chemicals were Cibachrome (Ciba-Geigy) and the printing procedure was based on the one recommended in the Cibachrome Color Print Manual (1975).

6. Light microscopy.

6.1 Observation.

A Zeiss Photomicroscope II, the same used for fluorescence microscopy, was employed for light microscopy except that the illumination was provided by a 60 W tungsten lamp and that the dark field condenser and objectives were replaced by ordinary ones (bright field observation). The usual film employed was Ektachrome 64 ASA which offered fine grain for high resolution.

6.2 Haematoxylin - Eosin staining.

The general procedure described in Humason (1972) was followed. Wax sections, 5 to 10 mm thick, were first deparaffinized in two successive xylene baths, 5 min each, hydrated in a succession of absolute ethanol baths (2 x 5 min), 70% ethanol (2 min), 30% ethanol (2 min) and double distilled water (5 min). The sections were then stained for 20 min with filtered haematoxylin (0.5%) Harris Haematoxylin; Mallory, 1944) and washed in tap water for 15 min. 30% and 70% ethanol immersion (5 and 3 min respectively) followed. Eosin (0.1% in 70% ethanol) (Humason, 1972) staining was done for 1 min. Dehydration was completed by successive immersion of the sections in 95% ethanol (1 min), absolute ethanol (2 x 5 min) and xylene (2 x 5 min). The slides were permanently mounted with Permount (Fisher Scientific Co.) or fluormount.

6.3 Thick Epon section staining.

To obtain better structural appearance and contrast, thick Epon section, 0.5 to 1.5 μm thick, were prepared from material fixed and embedded for electron microscopy (see later for detailed procedure). After sectioning on an ultramicrotome (Sorvall - Porter Blum MT-2), the Epon sections were transferred to a glass microscope slide. To attach it to the glass surface, the section was floated on a drop of water in the centre of the slide and heated at 70°C for 1 min on a hot plate

(Hayat, 1970). Richardson's staining procedure (Richardson, Jarett, and Finke, 1960) was then followed. It consisted of covering the Epon section with a 1:1 mixture of 1% Azure A in distilled water and 1% Methylene Blue in 1% Borax for 15 sec to 1 min, rinsing with water and drying on a warm plate.

7. Electron Microscopy.

7.1 Fixation and embedding.

Small fragments of the tissue to be examined under the electron microscope were dissected out with the blood circulation still intact. The tissue pieces were rinsed in cold 0.9% NaCl, cut into small blocks (1 mm^3) and fixed immediately at room temperature in a mixture of 4% formaldehyde (freshly prepared from paraformaldehyde), 2.5% glutaraldehyde and 1% acrolein in 0.1 M cacodylate buffer, pH7.3, for 2 hours (slightly modified from Dorey and Bhoola, 1972). Buffer rinsed tissue blocks were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour, dehydrated in a series of ethanol concentrations, ranging from 30% to absolute ethanol, immersed in pure propylene oxide (20 min), then in a 1:1 mixture of propylene oxide-Epon overnight before being embedded in Epon at 60°C for 48 hours.

7.2 Sectioning and Staining.

The Epon blocks were trimmed with a razor blade and thin sections (60 - 90 mk) were cut with an ultramicrotome. Thick Epon sections were first obtained to ascertain the presence and proper orientation of the material. The thin sections were placed on 200 mesh copper grids previously coated with a Formvar film (Ladd Research Industries, 0.25% Formar dissolved in ethylene dichloride), and stained by the double staining procedure (Pease, 1964). This consisted of inverting the grids on drops of 5% Uranyl acetate, in solution in methyl alcohol, placed on paraffin strips. These wax strips were placed in a Petri dish containing a filter paper soaked with 1N NaOH to absorb CO_2. This first staining lasted 10 min. The grids were then rinsed four times with double distilled water and stained with a freshly prepared lead citrate solution (Reynolds, 1963) for 10 min. The grids were allowed to dry on filter paper in a Petri dish before being examined in a Philips 300 electron microscope. Photographs were taken with the 35 mm camera incorporated in the microscope on Kodak fine grain positive film #5302. The film was processed using D-19 developer and Kodak fixer. Printing was done on various contrast grades of Kodabromide paper.

8. General Comments.

All experiments described in this work were performed at least twice, and in most instances three or four times, using tissue from different animals for each experiments.

If results were dubious, such as for the cat kidney and pancreas, no further investigations were undertaken after two or three unsuccessful attempts, judging that a considerable time might have to be devoted to get satisfactory results.

Immunohistochemical techniques, involving the use of horseradish peroxidase or ferritin labelled antibodies, were also tried in an attempt to find the ultrastructural localization of kallikrein in the cat's submandibular gland. Pre and post embedding immunohistochemical staining were both unsuccessful due to too high non-specific attachment of the antibodies to the embedding material and/or tissue components. Further investigation would be necessory to answer the important question which is the ultrastructural localization of kallikrein.

RESULTS

1. Guinea-Pig.

1.1 Coagulating gland.

1.1.1 Preparation and properties of antibody to CGK.

Out of six rabbits immunized with CGK, only three survived. Antibody was detected by immunodiffusion in the sera from two of the three surviving rabbits. The specificity of the antibody was suggested by the single precipitin arc observed. Typical results are illustrated in fig. 3. To test the specificity of the antibody for CGK, the serum was incubated with CGK and then tested again by immunodiffusion. As illustrated in fig. 4, partial inhibition was observed with 2.0 μg of CGK while complete inhibition occurred at and above 5 μg of CGK. Tests for specificity were also carried out using esterase activity as a measure of inhibition of CGK by the antibody present in the immune-serum. As shown in figure 5, after incubation of the CGK with the sera, only partial inhibition was observed with the immune-serum while no change occurred with the non-immune serum. This indicates that the antibody not only binds on the kallikrein molecule but does so in a way that interferes with its esterase activity. A further indication of the specificity of the antibody was obtained by incubating the immune-serum with CGK prior to immunofluorescence staining. This resulted in the complete disappearance of specific fluorescence. Contrary to what was observed for the CGK esterase activity, as seen in figure 6 no inhibition of the kininogenase activity occurred when CGK was preincubated with the immune-serum prior to testing its activity, using the guinea-pig's ileum as a

Fig. 3. Immunodiffusion of Guinea-pig Coagulating Gland Kallikrein.

The antigen (purified CGK) was tested against sera from blood collected before and after antigen injection (respectively called non-immune and immune serum). Numbers represent the concentration of antigen per 50 µl of solution. Each well received 50 µl of antigen solution or serum. Only immune serum showed reaction with the antigen, resulting in single precipitin arcs.
Top row: immune serum, bottom row: non-immune serum.

Fig. 3.

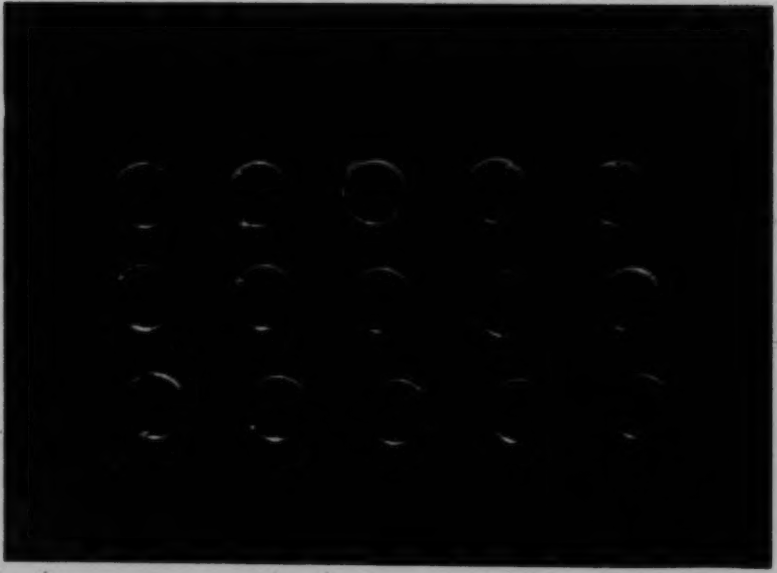
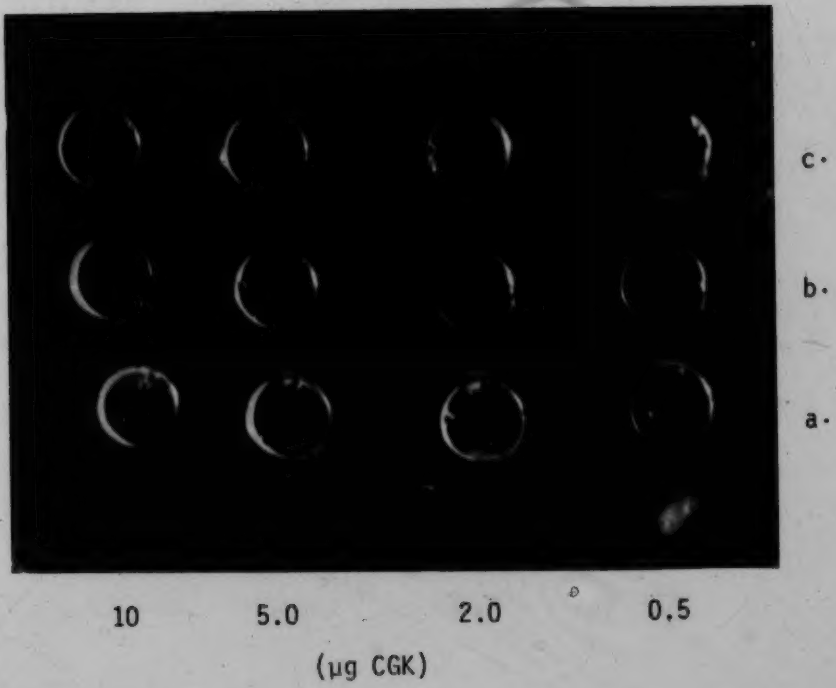

Ab
Ag
Ab
0.2
1.0
5.0
20
50

Fig. 4. Guinea-pig Coagulating Gland Kallikrein. Antigen-Antibody Inhibition Experiment: Immunodiffusion Test.

30 µl volumes of immune-serum were incubated for 15 min at room temperature with various amounts of CGK (0.5, 2.0, 5.0 and 10 µg). The resulting incubation mixture was then tested by immunodiffusion against a fixed amount of CGK (5 µg/50 µl). Aliquots (30 µl) of immune-serum were used as controls. Complete inhibition of the antibody by CGK occured at or above a concentration of 5 µg CGK/50 µl.

Fig. 4.


c.
b.
a.
10
5.0
2.0
0.5
(μg CGK)

a. Immune serum (30 μl)

b. CGK (5 μg/50 μl)

c. Incubated mixture (μg CGK/30 μl immune serum)

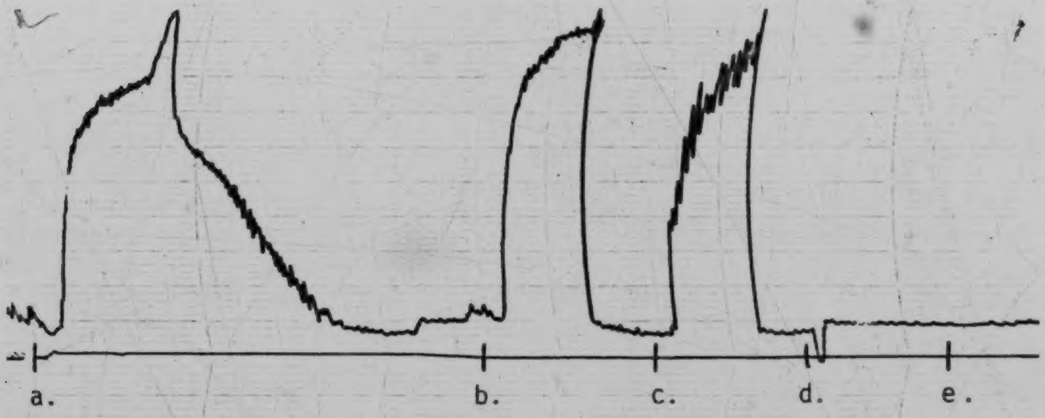
Fig. 5. Guinea-pig Coagulating Gland Kallikrein. Antigen-Antibody Inhibition Experiment : Esterase Activity.

Sample	Esterase Activity (mU)	% Inhibition
a.	34.5	--
b.	32.7	5.3%
c.	15.4	55.4%

a. CGK (5 μg) + 90 μl 0.9 NaCl (Control)
b. CGK (5 μg) + 90 μl non-immune serum
c. CGK (5 μg) + 90 μl immune serum

90 μl aliquots of immune and non-immune sera were incubated for 15 min at room temperature with 5 μg of CGK. The esterase activity of the resulting mixture was verified by the BAEe method of Trautschold (1970). A mixture of 5 μg CGK incubated with 90 μl of 0.9% NaCl was used as a control.

Fig. 6. Antigen-Antibody Inhibition Studies: Kininogenase Activity



a. CGK (5.0 µg) with 0.2 ml of substrate (50 mg/ml)

b. CGK (5.0 µg) with 0.2 ml of substrate pre-incubated with 100 µl immune serum

c. CGK (5.0 µg) with 0.2 ml of substrate pre-incubated with 100 µl non-immune serum

d. Non-immune serum (100 µl) with 0.2 ml of substrate

e. Immune serum (100 µl) with 0.2 ml of substrate

Various samples, as described above, were tested for their kininogenase activity by the biological assay preparation (Moriwaki and Schachter, 1971). When necessary, the sera were incubated with CGK for 15 min at room temperature. Dog pseudo-globulin was used as substrate.

biological assay preparation (Moriwaki and Schachter, 1971). It thus appears that the binding of the antibody to the kallikrein molecule was not interfering with its kininogenase activity, indicating a binding site for the antibody molecule different from the active site required for the release of kinin.

1.1.2 Histological observations.

As originally described by Walker (1910), the coagulating glands of the guinea-pig, considered a part of the prostate, consist of two elongated lobes which lie at the base of the seminal vesicles. Their muscular and fibrous framework is denser than for the prostate, the number of tubules less, the lumina of the alveoli larger and the villi more numerous. A single columnar epithelium lines the lumen of the gland of which the oval and rounded nuclei are at the base of the cell and the protoplasm is evenly granular. A section is illustrated in Plate 1 clearly showing the columnar epithelium surrounding the lumen.

1.1.3 Immunofluorescence.

1.1.3.1 Tissue fixation.

As described in materials and methods, various fixation techniques were tried. Sections of freeze-dried and frozen sections stained with haematoxylin-eosin had poor structural appearance so no further investigation was done with such sections. 95% ethanol as fixative (Sainte-Marie, 1962) resulted in the best structural appearance of the tissue and good preservation of the antigenicity of the tissue kallikrein. This property was demonstrated by immunodiffusion tests in which extracts of fresh gland and of gland fixed in ethanol

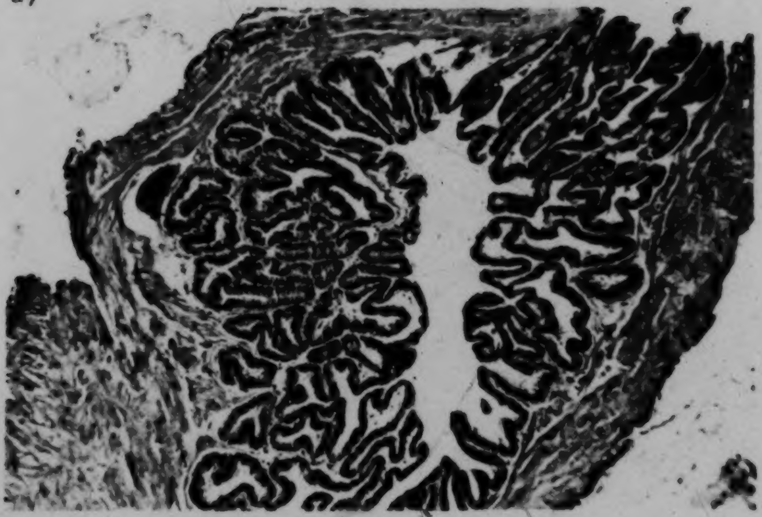
Plate I : Haematoxylin-Eosin Stained Sections of Guinea-pig Coagulating Gland.

a) Cross-sections of a lobule. Note the numerous villi. Original magnification: 25X.

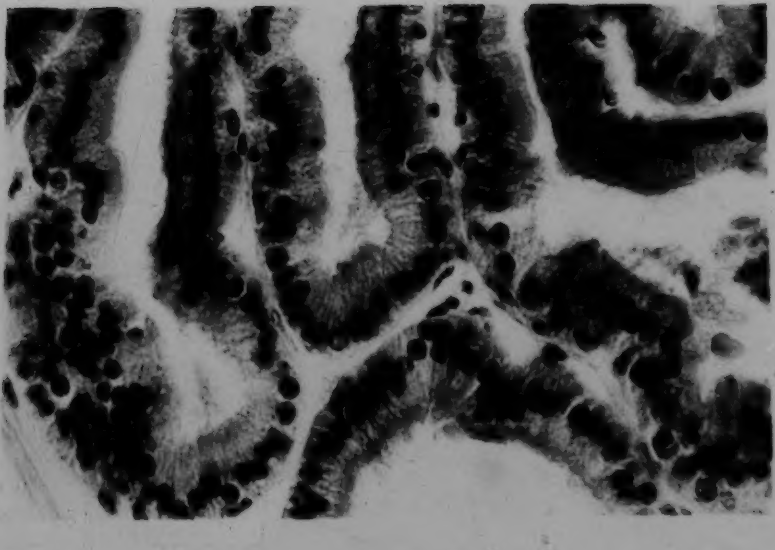
b) Single columnar epithelium lining the lumen of the gland. Original magnification: 160X.

Plate I.

a)



b)



were reacted with CGK-antibody. A single precipitin arc was obtained with two glands extracts. The same experiment was carried out with extract from glands fixed in ethanol, embedded in paraffin and cleared through xylene, ethanol and PBS. Again, immunodiffusion tests of these extracts with CGK-antibody resulted in single precipitin arcs, indicating preservation of the antigenicity of the tissue kallikrein through fixation and embedding. Immunofluorescence staining was therefore considered possible.

1.1.3.2 Tissue embedding and sectioning.

7 µm sections were cut from blocks embedded in paraffin, floated on water at 45°C and collected on albumized slides.

1.1.3.3 Immunofluorescence staining.

Serial tissue sections, cleared through xylene, alcohol and PBS, were disposed side by side on a glass slide and stained in such a way to obtain specific fluorescence in one section and three controls in the other area. First results, as pictured in plate II, were obtained using a Leitz Orthoplan microscope equipped with bright field condenser BG 38 and UGI excites and K 430 barrier filters. No counter staining was used and the incubation with the staining solutions was done in the cold for 18 hours.

The same experiments were repeated at a later date using a Zeiss Photomicroscope II fluorescence microscope equipped with dark field objectives and condenser, and a special FITC exciter filter.

Incubation of the sections was done at 37°C for 30 min and Evans blue was used as a counter stain. As seen in Plate III, the

Plate II : Localization of Kallikrein in Guinea-pig Coagulating Gland by Immunofluorescence Microscopy. Preliminary Results.

Bright field microscopy, 18 hours staining at 4°C and no counterstaining were used. Note the diffuse green specific fluorescence in all the secretory cells surrounding the crypts and lumen; only the nuclei, located in the basal portion of these cells, are non-reactive. The surrounding connective tissue shows no specific fluorescence. Original magnification: 160X.

Plate II.

Plate III : Localization of Kallikrein in Guinea-pig Coagulating Gland by Immunofluorescence Microscopy. Later results.

Dark field microscopy, 30 min staining at 37°C and Evans blue counterstaining were used.

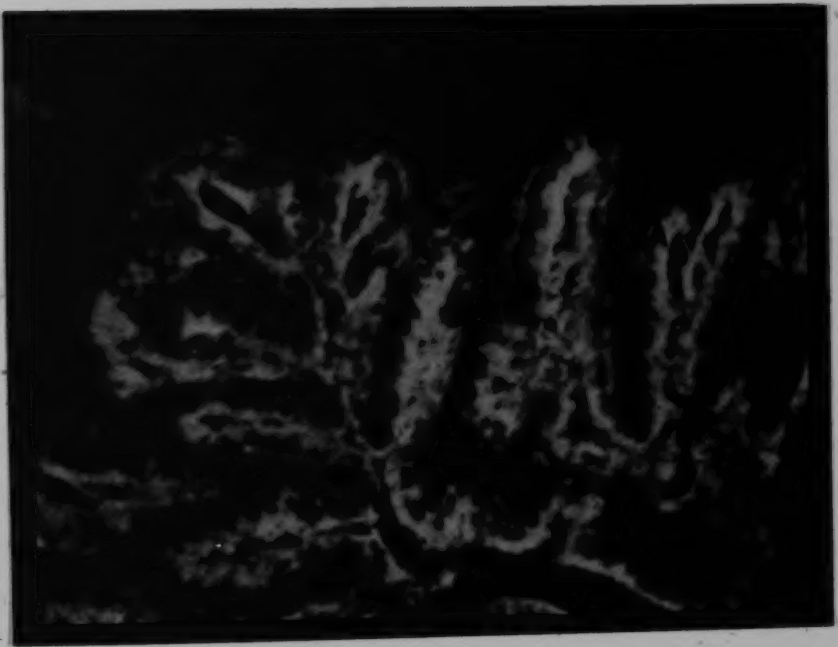
a) Control tissue section without addition of antibody to kallikrein. Original magnification: 65X.

b) Tissue section after reaction with antibody to kallikrein. Note the sharper contrast due to dark field microscopy and counterstaining. Original magnification: 65X.

Plate III.

a)

b)



contrast is higher due to dark field microscopy and the specific fluorescence can be observed more easily due to the red fluorescence of the background tissue conferred by the counterstaining. Thereafter, this last described procedure was followed for the vast majority of immunofluorescence staining experiments.

1.2 Prostate glands.

Since it had been reported that kallikrein was also present in guinea-pig's prostate gland (Bhoola et al., 1962, Moriwaki & Schachter, 1971), it was believed to be worthwhile to test for the presence of kallikrein by immunofluorescence.

1.2.1 Histological observations.

The prostate gland of the guinea-pig consists of four lobes, two dorsal and two ventral ones, lying next to the coagulating glands, at the base of the seminal vesicles (Walker, 1910). These lobes are made up of tubules lined by a single layer of columnar epithelium, each communicating with the urethra by a single duct. Haematoxylin-eosin stained sections are illustrated in plate IV. In general, a structure similar to coagulating gland is seen (see plate I) except that the number of villi is smaller than in the coagulating gland.

1.2.2 Immunofluorescence.

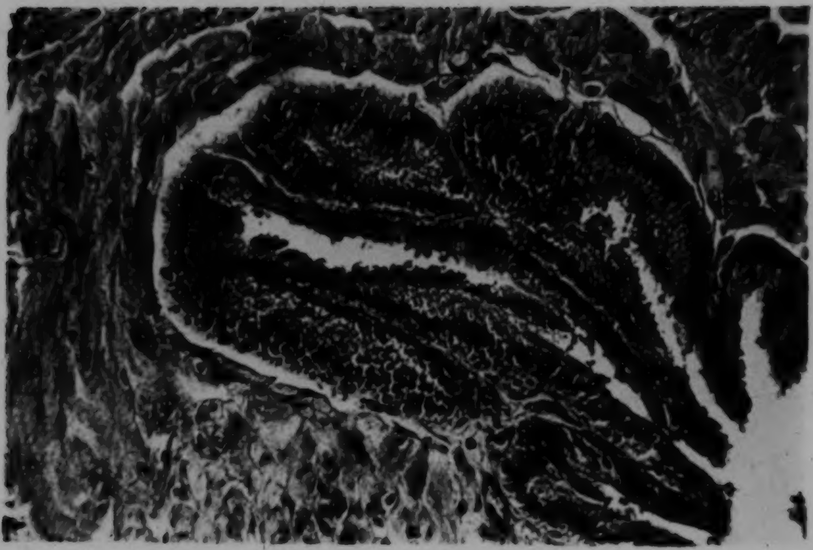
The standard immunofluorescence staining technique was followed, except that in some instances the incubation time was doubled (to 60 min) to give more time for the antibody to react with the low concentration of kallikrein found in that tissue.

There was no difference between the control, in which non-immune serum was used, and the test section, indicating that the

Plate IV : Haematoxylin-Eosin Stained Sections of Guinea-pig Prostate.

Cross-sections of a gland lobule. Note the low number of villi compared to coagulating gland (plate 1). Original magnification: 65X.

Plate IV.



kallikrein present in the prostate either is in too low concentration or has different antigenic properties than the CGK.

1.3 Guinea-pig sperm.

Since acrosin, an enzyme with the specificity and potency of trypsin, found in the acrosomal portion of the spermatozoid, is believed to belong to the class of kininogenases (Fritz, 1975), it was of interest to determine if acrosin would be revealed by immunofluorescence with antibody toward CGK. Both sperm smears and epididymidis sections, however, failed to show any indication of such fluorescence. Since the acrosomal membrane might act as a barrier not allowing the antibody to reach the enzyme in the acrosome, sperm samples were frozen and thawed and many acrosomes were partly disrupted in smears prepared from these sperm samples but still no specific fluorescent staining was observed.

1.4 Submandibular gland.

Since it had been observed that different kallikreins in the rat are immunologically cross-reactive with one another (Brandtzaeg, et al., 1976), we thought that the same might be true for guinea-pig's submandibular kallikrein and CGK. First, immunofluorescence was tried on the submandibular gland, using immune-serum for CGK. The assumption that cross-antigenicity existed between these two sources of kallikrein was supported by the ductal localization of fluorescence as in the rat (Orstavik et al., 1975).

1.4.1 Histological observations.

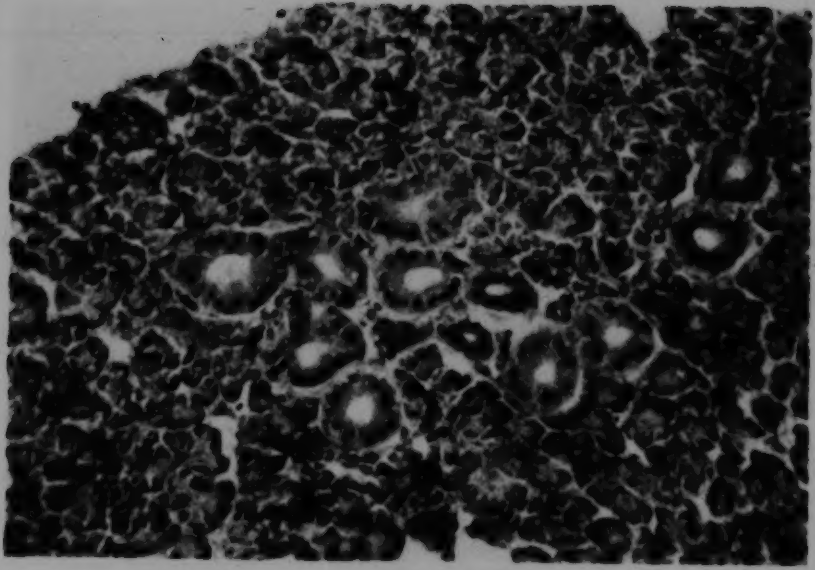
Plate V shows haematoxylin-eosin stained sections. The acini and the duct cells can be easily distinguished. The duct system

Plate V : Haematoxylin-Eosin Stained Sections of Guinea-pig Submandibular Gland.

Note the numerous striated ducts, noticeable by their single columnar epithelium lining the lumen.

Original magnification: 65X.

Plate V.



consists of short intercalated ducts, made of small squamous cells, joining the acinar cells to the striated ducts which are lined with single layer of columnar epithelium. This intralobular duct system becomes interlobular and merges finally into a main collecting or main excretory duct which is lined with stratified epithelium consisting mainly of cuboidal cells.

1.4.2 Immunofluorescence.

1.4.2.1 Staining of normal glands.

Plate VI shows specific fluorescence in the test section compared to the control treated with non-immune serum. The fluorescence in the apical portion of the striated duct cells corresponds to the location of kallikrein in the cat's submandibular gland (see later).

1.4.2.2 Ligation experiments.

As illustrated in fig. 7, a seven day duct ligation resulted in almost complete depletion of kallikrein in the gland. These results were confirmed by immunofluorescence since practically no fluorescence could be detected after a seven day ligation. These observations are in accord with ligation experiments in the cat (Beilenson et al., 1968, Barton, et al., 1975).

1.5 Comparative studies: Coagulating, prostate and submandibular glands.

1.5.1 Kallikrein content.

As shown in fig. 8, relatively high activity was found in extracts of coagulating and submandibular glands while little activity was present in prostate gland extract.

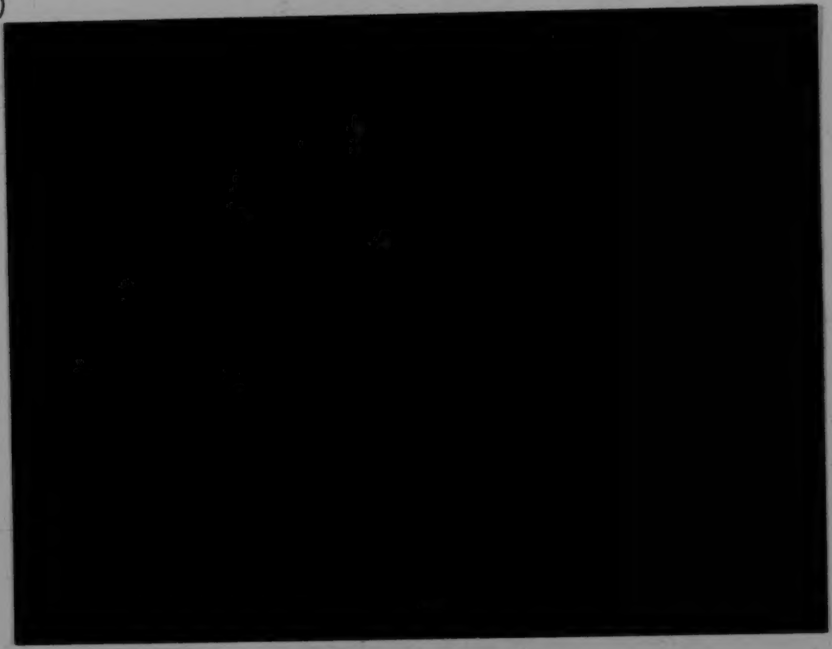
Plate VI : Localization of Kallikrein in Guinea-pig Submandibular Gland by Immunofluorescence Microscopy.

a) Control tissue section without addition of antibody to kallikrein. Serial section preceeding b) below. Only slight and non-specific fluorescence is observed throughout. Original magnification: 65X.

b) Tissue section after reaction with antibody to coagulating gland kallikrein. Note the intense specific fluorescence located exclusively in the striated ducts, and concentrated near the luminal border of striated duct cells. Original magnification: 65X.

Plate VI.

a)



b)

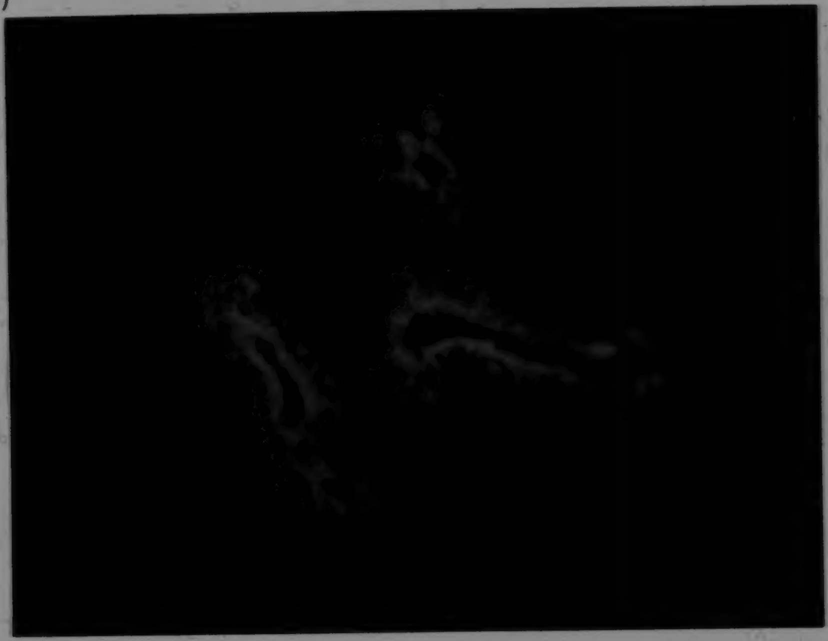


Fig. 7. Kallikrein Content in Ligated Submandibular Gland of the Guinea-pig.

	Esterase Activity (mU/mg)		Kininogenase Activity (µg bradykinin eq./mg)	
	Control -	Ligated	Control -	Ligated
Guinea-pig 1	23	2.6	2.0	0.12
Guinea-pig 2	18	2.3	1.3	0.10

The main excretory duct of the left submandibular gland of two guinea-pigs was ligated. Glands were removed after seven days, the contralateral gland serving as a control. Esterase activity was determined by the method of Trautschold (1970) using BAEe as substrate. The biological assay preparation (Moriwaki and Schachter, 1971) was used to verify the kininogenase activity. Activities are expressed in units per mg of freeze-dried gland.

Fig. 8. Kallikrein Content of Guinea-pig's Coagulating, Prostate and Submandibular Glands.

Gland Extract	Esterase Activity (mU/mg)	Kininogenase Activity µg bradykinin eq./mg
Coagulating gland	130	0.90
Prostate gland	3	0.06
Submandibular gland	55	--

Gland extracts were prepared from freeze-dried glands and their activities measured by the BAEe method of Trautschold (1970) for esterase activity and the biological assay preparation (Moriwaki and Schachter, 1971) for kininogenase activity. Units of activity are expressed per mg of freeze-dried gland. Not enough gland extract was available to determine the kininogenase activity of the submandibular gland.

1.5.2 Cross-antigenicity and specificity of CGK.

From the same gland extracts prepared (see preceeding section), aliquots were used to test the antigenicity of the kallikrein present in these extract with CGK-antibody. However, the prostate gland had such a low activity that it was found impossible to prepare an extract having a sufficiently high activity to test it. Thus, only coagulating and submandibular glands were tested.

1.5.2.1 Immunodiffusion.

Coagulating gland and submandibular gland extracts were tested by immunodiffusion versus immune-serum containing CGK-antibody. As illustrated in fig. 9, only coagulating gland extract reacted and formed single precipitin arcs.

1.5.2.2 Kininogenase activity.

To verify the effect of CGK-antibody on the inhibition of the kininogenase activity of the submandibular gland extract, immune-serum was pre-incubated with guinea-pig submandibular gland extract prior to performing a determination of kininogenase activity by the biological assay. As shown in fig. 10, no significant difference was observed between control (immune serum replaced by PBS) and the test sample.

1.5.2.3 Immunofluorescence.

To verify the specificity of the CGK-antibody toward the kallikrein contained in the submandibular gland and to show the cross-antigenicity between CGK and submandibular kallikrein, CGK was incubated prior to immunofluorescence staining with the immune-serum containing the antibody specific for CGK. This resulted, in complete

Fig. 9. Immunodiffusion of Coagulating and Submandibular Gland Extracts Versus Immune Serum Containing CGK-Antibody.

#1. Coagulating gland extract - 14.8 mU/trough
#2. Coagulating gland extract - 29.6 mU/trough
#3. Coagulating gland extract - 44.4 mU/trough
#4. Submandibular gland extract - 47.1 mU/trough
#5. Submandibular gland extract - 15.7 mU/trough
#6. Submandibular gland extract - 31.4 mU/trough
#7. Submandibular gland extract - 47.1 mU/trough
#8. Coagulating gland extract - 44.4 mU/trough

Immunodiffusion test was performed between coagulating and submandibular gland extracts versus immune serum (Ab) containing antibody specific for coagulating gland kallikrein. Wells were filled with 30μl of the various solutions described above.

Fig. 9.

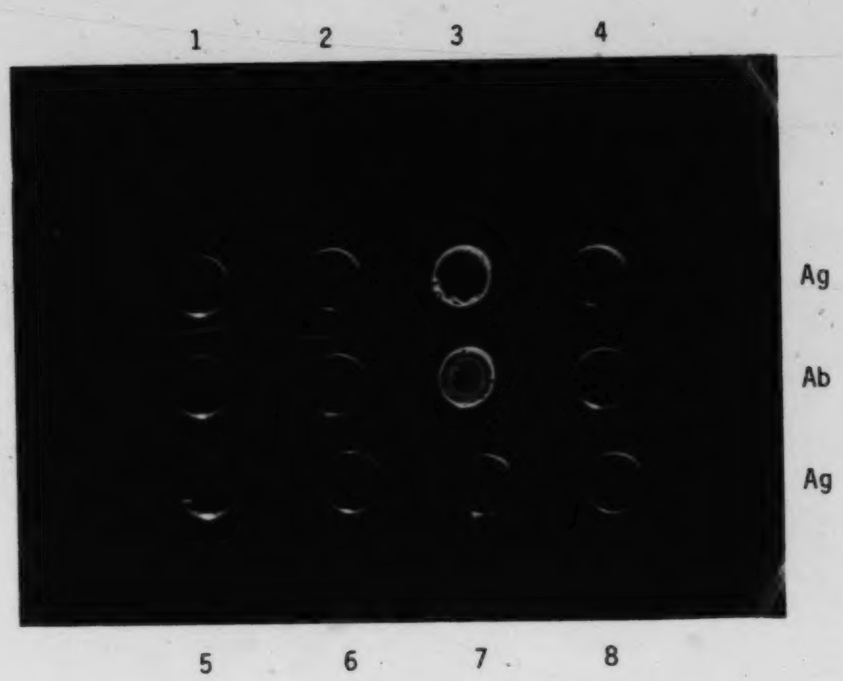
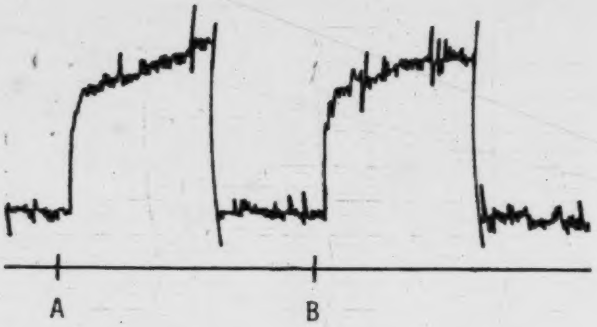

1
2
3
4
Ag
Ab
Ag
5
6
7
8

Fig. 10. Effect of CGK Antibody on the Kininogenase Activity of the Guinea-pig's Submandibular Gland Extract.



Immune serum (100 µl) containing antibody specific for guinea-pig's coagulating gland kallikrein (sample A) and PBS pH 8.0 (100 µl) (sample B) were incubated for 15 min at room temperature with 25 µl of guinea-pig's submandibular gland extract containing the equivalent of 1.0 mg of freeze-dried gland per ml. Procedure followed was as described by Moriwaki and Schachter (1971).

absence of specific fluorescence in submandibular gland sections. Control sections, in which CGK was replaced by PBS showed the usual intense specific fluorescence. Similar results were obtained if coagulating gland sections were stained with immune-serum preincubated with CGK. These results support the view that the antigenicity of the kallikrein present in the ducts of submandibular gland is similar or close to the one present in the coagulating gland of the guinea-pig.

1.5.3 Castration experiments.

Samples of glands from 3 week-castrated and controls animals were prepared for immunofluorescence staining while the rest of the glands were excised and freeze-dried for esterase activity determination. No significant difference in fluorescence was observed between glands of castrated or control animals. These results correlated well with the esterase values of the excised glands (fig. 11).

2. Cat.

2.1 Cat salivary kallikrein and its antibody.

Cat salivary kallikrein (CSK) was kindly provided by Dr. Moriwaki who had purified it and tested its purity in his laboratory (Moriwaki, Hojima and Schachter, 1976; see Methods).

2.1.1 Preparation and properties of antibody to CSK.

Since relatively a small amount of purified CSK was available (5.0 mg), only two rabbits were injected with this antigen. Only one of the two rabbits responded immunologically to the injection, the specificity of antibody to CSK being suggested by single precipitin

Fig. 11. Effect of Castration on Kallikrein Content of Guinea-pig's Coagulating and Submandibular Glands.

	Esterase Activity (mU/mg)		
	Submandibular Gland		Coagulating Gland
	Left -	Right	
Guinea-pig 1	77	88	87
Guinea-pig 2	66	76	38
Guinea-pig 3	21	35	52

Two guinea-pigs (#2 and 3) were castrated and their right submandibular glands excised and freeze-dried. A third guinea-pig (#1) had also its right submandibular gland removed but was not castrated. Three weeks after, the left submandibular and coagulating glands of all animals were excised, freeze-dried and the esterolytic activity of the extracts prepared determined by the BAEe method of Trautschold (1970).

arcs in the immunodiffusion test (fig. 12).

To further study the properties of the antibody found in the immune-serum, series of inhibition tests were performed and verified by a) immunodiffusion, b) chemical (esterase activity) and c) biological (kininogenase activity) tests.

a) Immunodiffusion.

Immune-serum containing antibody was incubated with varying concentrations of CSK. Complete inhibition, between our CSK preparation and its antibody resulted at or above 2.0 μg CSK (fig. 13).

b) Esterase activity.

As shown in fig. 14, partial inhibition of esterase activity occurred after incubation with the sera. Comparing results between CSK incubated with immune serum and with non-immune serum, an inhibition of 20.0% is noticed with the presence of antibody. There might thus be some interference between the antibody binding site and the esterase activity site on the CSK molecule.

c) Kininogenase activity.

Inhibition of kininogenase activity was observed by incubating CSK with its antibody prior to performing biological assays (Moriwaki and Schachter, 1971). CSK alone, and mixtures of CSK with immune and non-immune sera were tested for kininogenase activity. As illustrated in fig. 15, and contrary to what had been observed for guinea-pig's coagulating gland kallikrein, there was complete inhibition of the CSK kininogenase activity by its antibody and there was no inhibition by the non-immune serum. There thus appears to be overlap of the antibody binding site and the kininogenase activity

Fig. 12. Immunodiffusion of Cat Submandibular Gland Kallikrein.

The antigen (purified CSK) was tested against serum from blood collected from rabbits injected with CSK. Each well received 40 µl of solution. The figures represent the amount in µg of antigen per well.

Fig. 12.

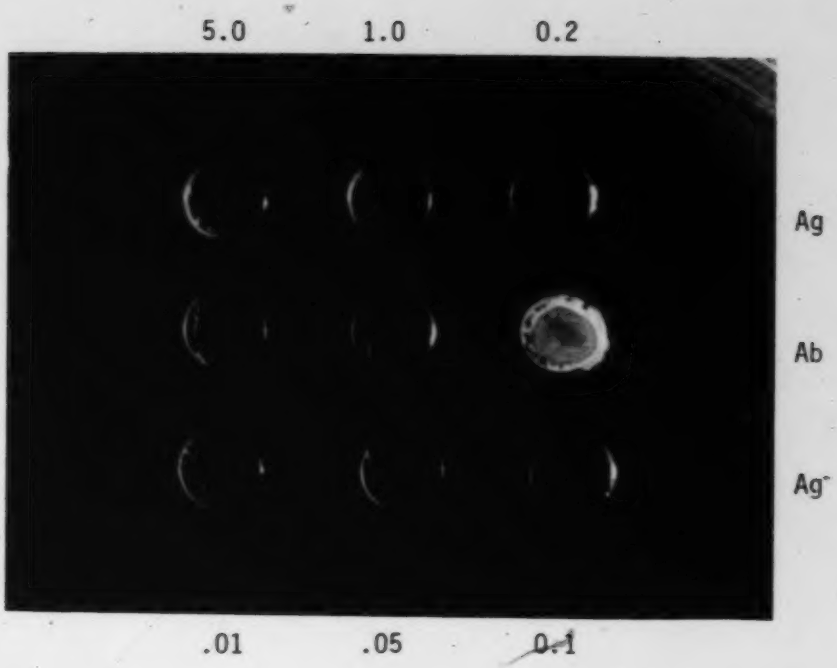
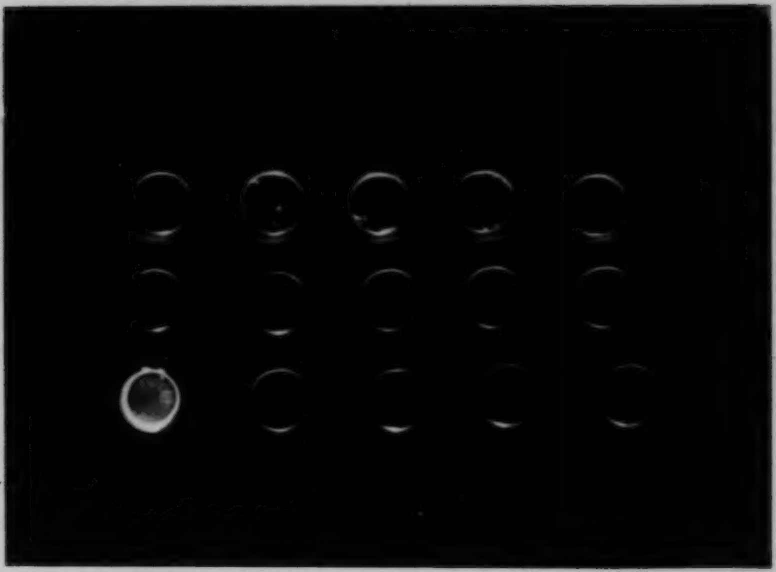

5.0
1.0
0.2
Ag
Ab
Ag
.01
.05
0.1

Fig. 13. Cat Submandibular Gland Kallikrein. Antigen-Antibody Inhibition Studies: Immunodiffusion Test.

40 µl aliquots of immune serum were incubated for 15 min at room temperature with various amounts of CSK (.05, 0.2, 0.5, 2.0 and 5.0 µg). The resulting incubated mixture has been tested by immunodiffusion against a fixed concentration of CSK (1.0 µg/40 µl). 40 µl samples of immune serum were used as controls. Complete inhibition occured at and above a concentration a concentration of 2.0 µg CSK/40 µl.

Fig. 13.


a.
b.
c.
.05
0.2
0.5
2.0
5.0

a. Immune serum (40 µl)

b. CSK (1.0 µg)

c. Incubated mixture (µg CSK/40 µl immune serum)

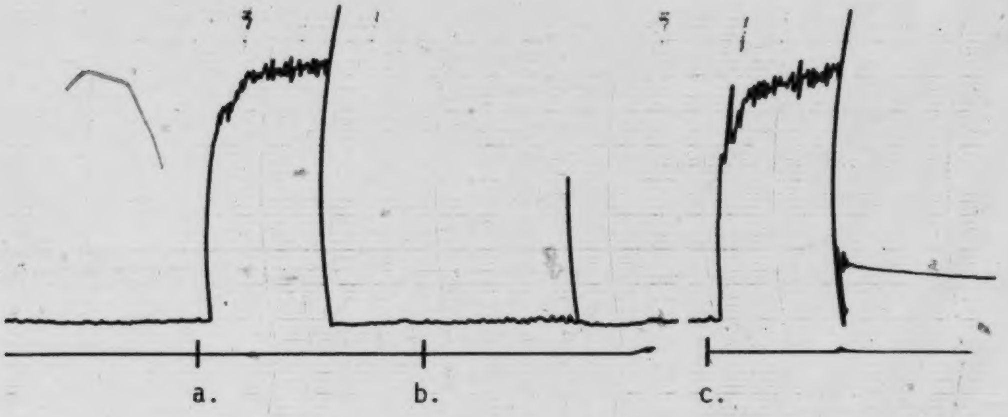
Fig. 14. Cat Submandibular Kallikrein. Antigen-Antibody Inhibition Studies: Esterase Activity.

Sample	Esterase Activity (mU)	% INhibition
a.	16.1	--
b.	12.7	20.5%
c.	10.0	37.9%

a. CSK (0.67 μg) (control)
b. CSK (0.67 μg) + 33 μl non-immune serum
c. CSK (0.67 μg) + 33 μl immune serum

100 μl aliquots of immune and non-immune sera were incubated for 15 min at room temperature with 2.0 μg of purified CSK. The esterase activity of the resulting mixture was verified by the BAEe method of Trautschold (1970). Control consisted of 2.0 μg of CSK incubated with 100 μl of 0.9% saline. 50 μl of the incubated mixtures were used for esterase activity determination.

Fig. 15. Cat Submandibular Kallikrein. Antigen-Antibody Inhibition Studies: Kininogenase Activity.



a. CSK (5.0 μg)

b. CSK (5.0 μg) incubated with immune serum (100 μl)

c. CSK (5.0 μg) incubated with non-immune serum (100 μl)

The kininogenase activity of various samples was tested by the biological assay preparation (Moriwaki and Schachter, 1971). In b. and c., the submandibular kallikrein was incubated with appropriate serum for 15 min at room temperature prior to activity determination.

site on the CSK molecule. The specificity of the antibody contained in the immune-serum toward CSK was further supported by the complete disappearance of specific fluorescence if CSK was incubated with the immune-serum prior to immune fluorescence staining.

2.2 Submandibular gland.

2.2.1 Histological observations.

2.2.1.1 Haematoxylin-eosin staining.

The typical configuration of haematoxylin-eosin stained sections is illustrated in plate VII, showing the various cell types, mainly mucous acinar and demi-lune and striated duct cells (Shakleford and Klapper, 1962; Shakleford and Wilborn, 1968).

2.2.1.2 Thick Epon sections.

To obtain better resolution under light microscopy, thick Epon section (0.5 - 1.5 µm) were cut from blocks prepared for electron microscopy (see next paragraph). As seen in plate VIII, the structural appearance of such a preparation is more detailed than in wax sections, revealing subcellular components such as acinar and demi-lune granules which can hardly be resolved in wax sections. The preparation of such sections also allowed a pre-selection of the tissue blocks for electron microscopy, ascertaining the presence of the desired features in the ensuing thin sections.

2.2.1.3 Electron microscopy.

Plate IX is a typical illustration of cat submandibular gland sections clearly showing the acinar and demi-lune cells and their subcellular components. Plate X shows in detail a cross-section of a striated duct where secretory granules are particularly evident. A

Plate VII : Haematoxylin-Eosin Stained Sections of Cat Submandibular Gland.

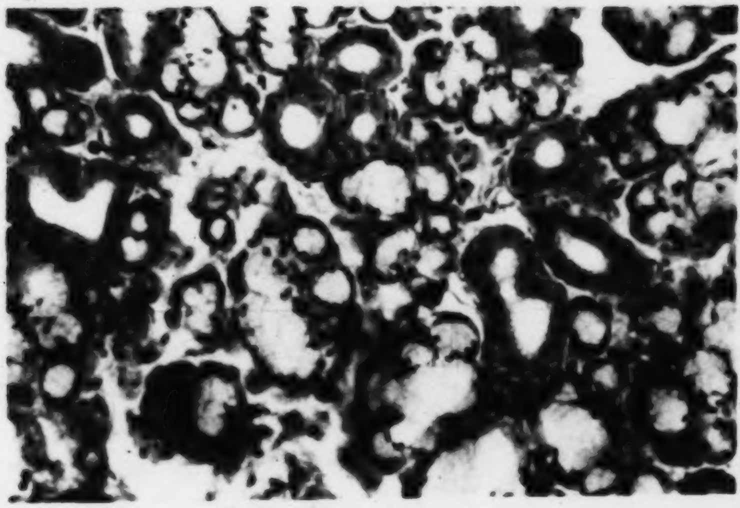
Note the mucous acinar cells capped with demi-lune cells. The striated ducts are noticeable by their regular round or oval appearance and the single columnar epithelium lining the lumen.

a) Note the numerous striated ducts. Original magnification: 65X.

b) Higher magnification showing clearly the differences between acinar, demi-lune and striated duct cells. Original magnification: 160X.

Plate VII.

a)



b)

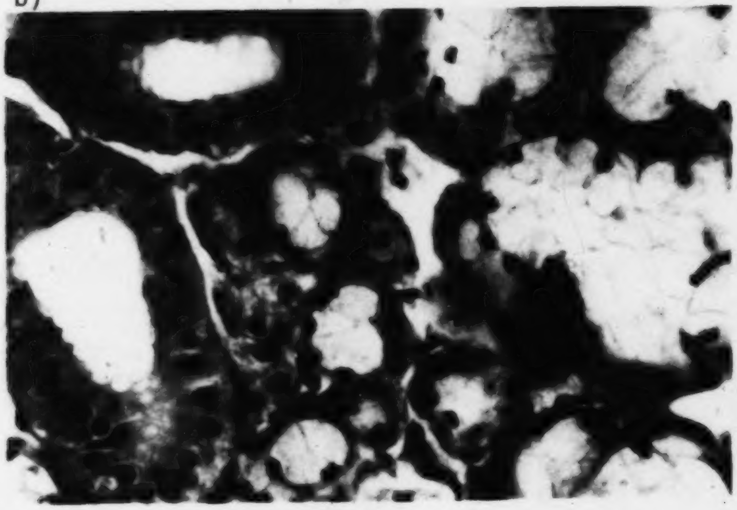


Plate VIII : Thick Epon Tissue Section of Cat Submandibular Gland.

High magnification of section of submandibular gland embedded in Epon and stained with Richardson's stain (Richardson et al., 1960). Note the opaque appearance of the granules of the acinar cells compared to the clear granules of the demi-lune cells. Striated duct cell granules, densely concentrated in the apical portion of the cells, are just barely noticeable. Striated duct is on the left, a demi-lune on the center and an acinar cell on the right of the picture. Original magnification: 400X.

Plate VIII.

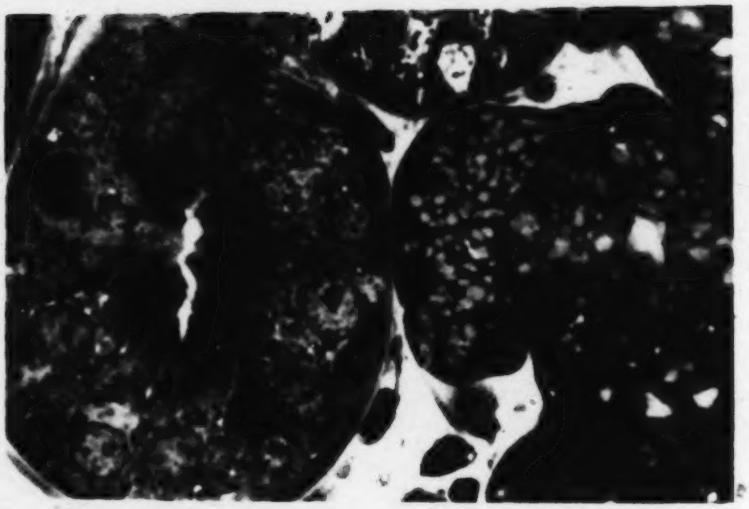


Plate IX : Electron Micrographs of Cat Submandibular Gland Sections: Acinar and Demi-lune Cells.

Micrographs showing in details the structural appearance of acinar and demi-lune cells granules. Both types of granules have approximately the same size (1-5 µ) but differ by their opacity, the demi-lune granules having a clear appearance compared to acinar granules.

a) Low magnification of acinar (top) and demi-lune cells (bottom). Original magnification: 1617X.

b) Demi-lune cell granules. Note the dark inclusion body inside some of the granules. Original magnification: 7420X.

c) Acinar cell granules. Note variation in opacity. Original magnification: 7420X.

Plate IX.

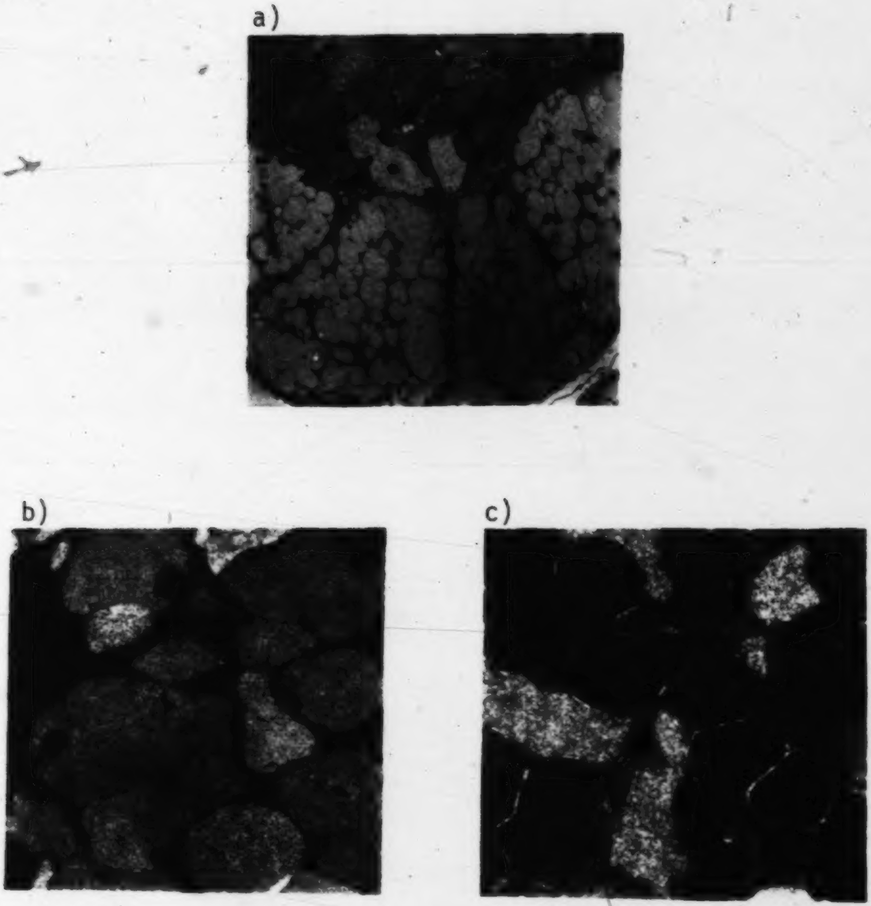


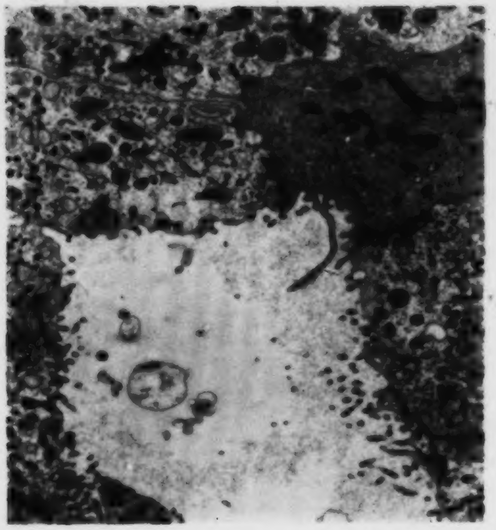
Plate X : Electron Micrographs of Cat Submandibular Gland Sections: Striated Duct Cells.

a) Striated duct cells surrounding a lumen. Note the dark cell in top right hand corner, free of secretory granules. Such secretory granules can be observed in light cells. Original magnification: 1617X.

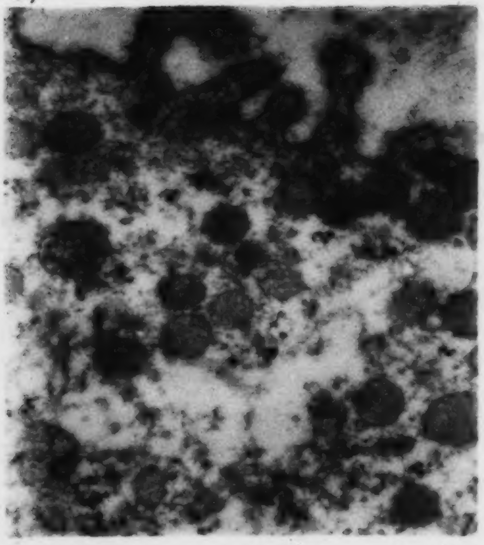
b) Details of the apical portion of a striated duct cell. Note the secretory granules close to the lumen, which is at the top of the picture. Original magnification: 15,362X.

Plate X.

a)



b)



clear difference can be noticed between acinar, demi-lune and striated duct cells granules due to their size, their density and/or inclusion of a dense body (demi-lune granules). Plate XI shows the ultrastructure of the stratified epithelium lining the lumen of the interlobular collecting duct system where again, secretory granules, of the same type observed in striated duct cells, can be seen.

2.2.2 Immunofluorescence.

After experience with the guinea-pig's coagulating gland, a rapid and satisfactory procedure for tissue fixation, embedding, sectioning and staining was established and used thereafter in the cat and dog (see Methods).

The cat's submandibular gland was a good tissue to work with, being easy to section and showing intense specific fluorescence. As shown in plate XII, the fluorescence is mainly located in the apical or luminal portion of the striated duct cells. A faint auto-fluorescence only could be observed in the other structures of the gland. This excludes demi-lune and acinar cells as a source of submandibular gland kallikrein. Fluorescence was also observed in larger interlobular collecting ducts (Plate X111). The fluorescence in this case was also located in the apical portion of the first layer of cells constituting the stratified epithelium lining the lumen in a location corresponding to the secretory granules observed in these cells under electron microscopy (see plate XI). These observations are thus similar to the results obtained in the guinea-pig's submandibular gland.

2.3 Sublingual gland.

2.3.1 Histological observations.

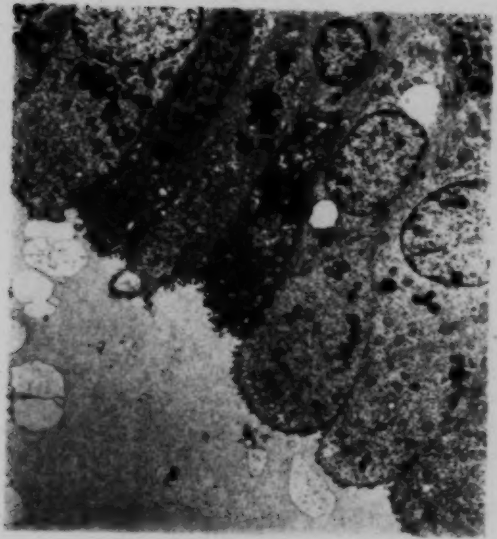
Plate XI : Electron Micrographs of Cat Submandibular Gland Sections: Stratified Epithelial Ducts.

a) Note the alternance of light and dark cells, the latter being devoied of secretory granules. Original magnification: 924X.

b) Details of the apical portion of a stratified epithelial duct cell. Note the similarity of the granules observed here with the one in the striated duct cell (plate X). Original magnification: 11,130X.

Plate XI.

a)



b)

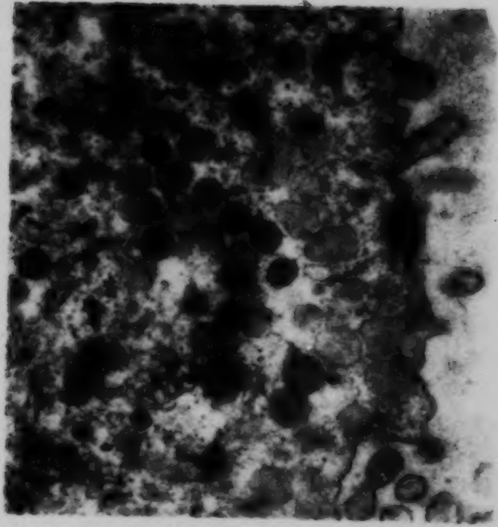


Plate XII : Localization of Kallikrein in Cat Submandibular Gland by Immunofluorescence Microscopy: Striated Ducts.

a) Control tissue section prepared without addition of antibody to kallikrein. Note absence of any fluorescence in acinar cells which appear black and the mild diffuse fluorescence of striated ducts showing little or no contrast with surrounding tissues. Original magnification: 65X.

b) Tissue section after reaction with antibody to kallikrein. Note the intense band of yellow-green fluorescence in the apical region of striated duct cells. Fluorescence is absent in acinar and demilune cells as well as in surrounding tissue. Original magnification: 65X.

c) High magnification of a single striated duct. Note the concentration of fluorescence close to the lumen. Original magnification: 400X.

Plate XII.

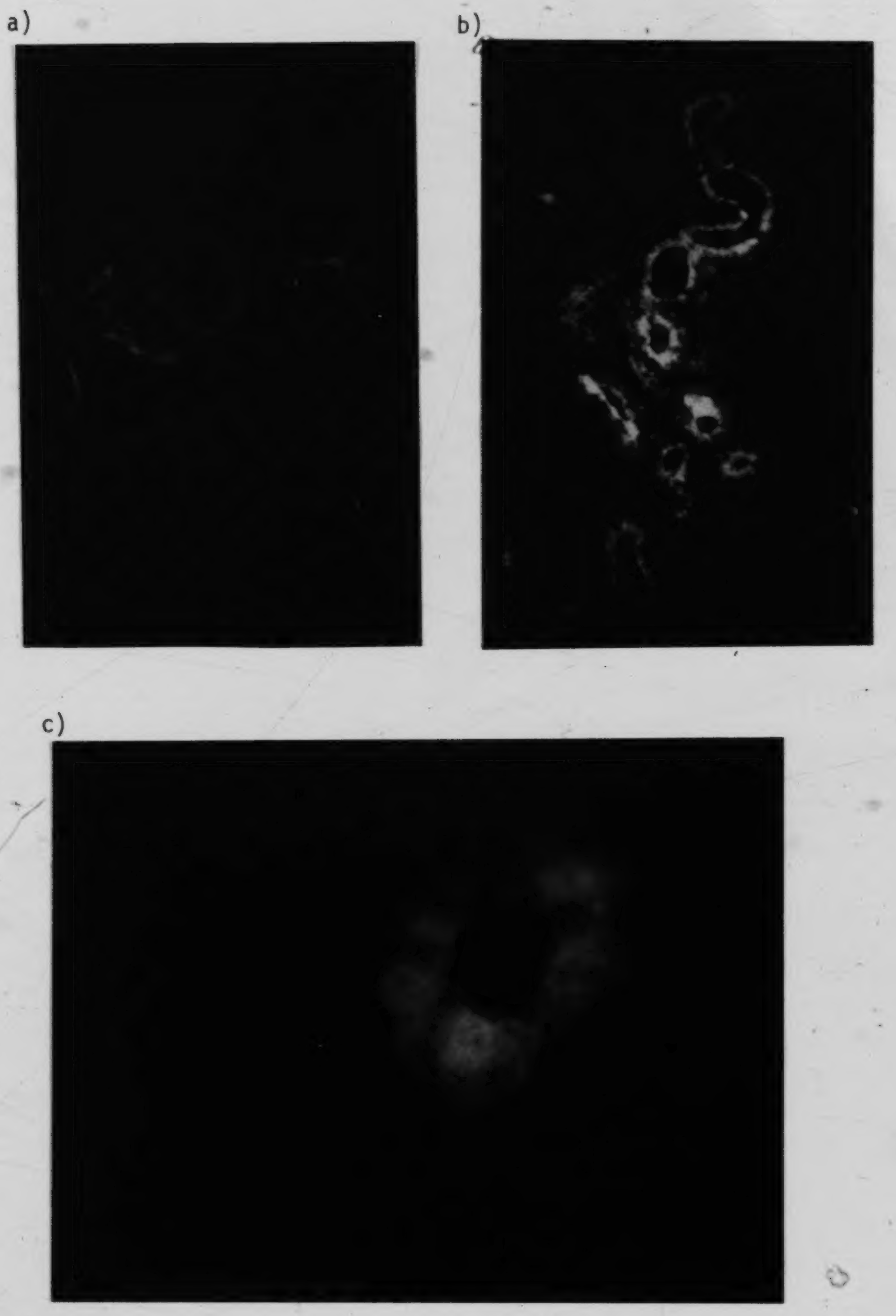


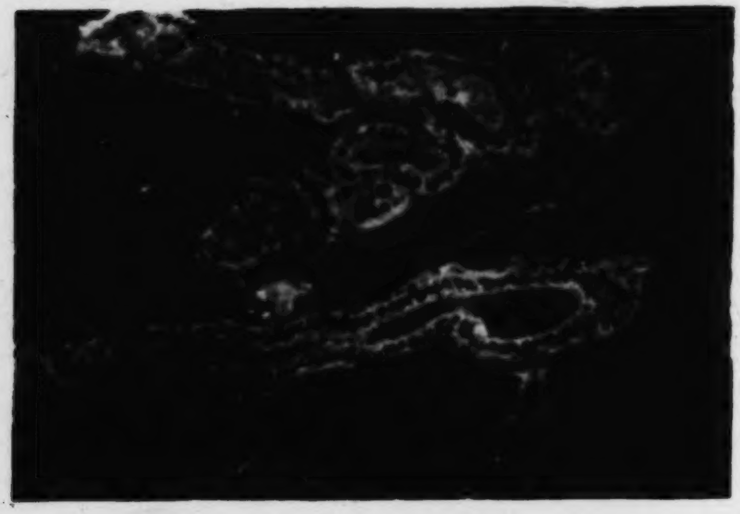
Plate XIII : Localization of Kallikrein in Cat Submandibular Gland by Immunofluorescence Microscopy: Collecting Ducts.

a) Control tissue section prepared without addition of antibody to kallikrein. Note diffuse fluorescence throughout, particularly noticeable in duct cells. Original magnification: 65X.

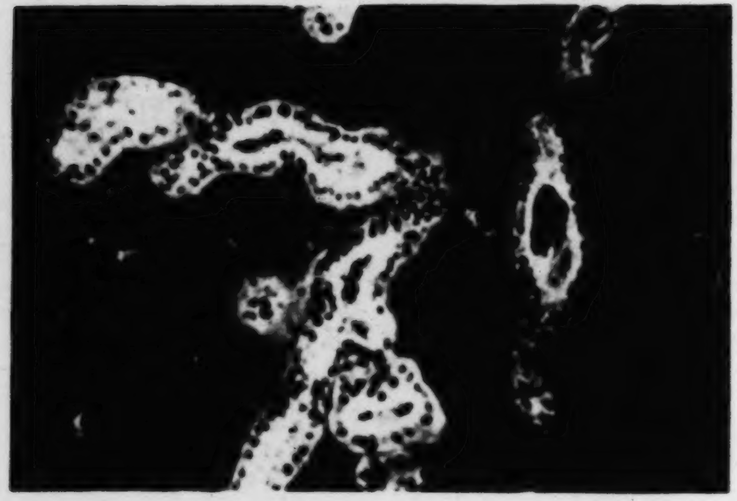
b) Tissue section after reaction with antibody to kallikrein. Note the intense specific fluorescence in the apical portion of the duct cells. Original magnification: 65X.

Plate XIII.

a)



b)



As described by Schackleford and Wilborn (1968) and Tandler and Poulsen (1977), the sublingual glands, are composed of special serous acinar cells, myo-epithelial cells and a duct system. The latter structure is composed primarily of mucous tubules and almost no striated ducts seem to be present. This is illustrated in plate XIV, a haematoxylin-eosin stained section. Surprisingly, some sections showed a different structure, rather similar to submandibular gland, containing acinar and demi-lune cells and striated ducts (see plate VII). This result will be discussed later.

2.3.2 Immunofluorescence.

Most sections failed to show any specific fluorescence (plate XV), except, occasionally, when sections contained striated ducts which did fluoresce. Examination of the entire sublingual gland accounted for this inconsistency by revealing a very uneven distribution of the few striated ducts it contained. A few lobules, usually peripheral ones, contained many striated ducts. Little or no specific fluorescence was observed in the stratified interlobular collecting or main ducts.

2.4 Parotid gland.

2.4.1 Histological observations.

The parotid glands are similar to sublingual glands in the small number of striated ducts they contain. They are comprised mainly of seromucous acini as described by Schackleford and Wilborn (1968). A typical haematoxylin-eosin stained section is illustrated in plate XVI.

2.4.2 Immunofluorescence.

Immunofluorescence staining was done in the usual way with

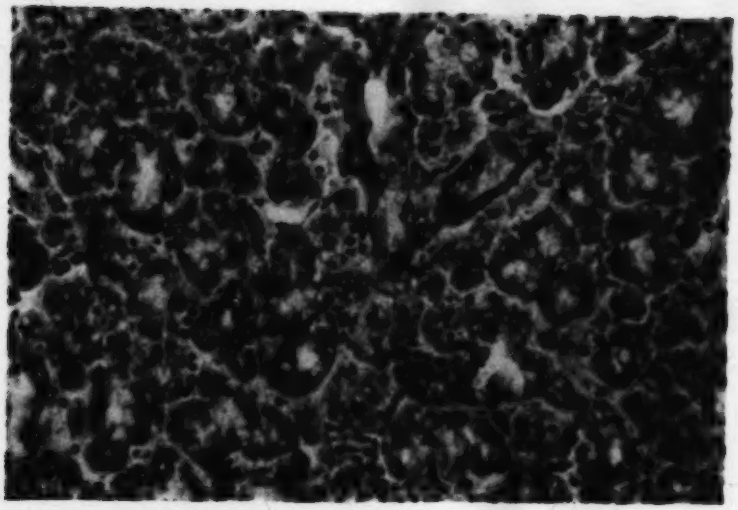
Plate XIV : Haematoxylin-Eosin Stained Sections of Cat Sublingual Gland.

a) Section showing typical structure of most of the gland. Note absence of any striated ducts. Original magnification: 65X.

b) Sections of some peripheral lobules of the sublingual gland showing numerous striated ducts, acini and demi-lune cells. Since the structure of such lobules is very similar to submandibular gland, it is possible that this section came from this latter organ (see text). Original magnification: 65X.

Plate XIV.

a)



b)

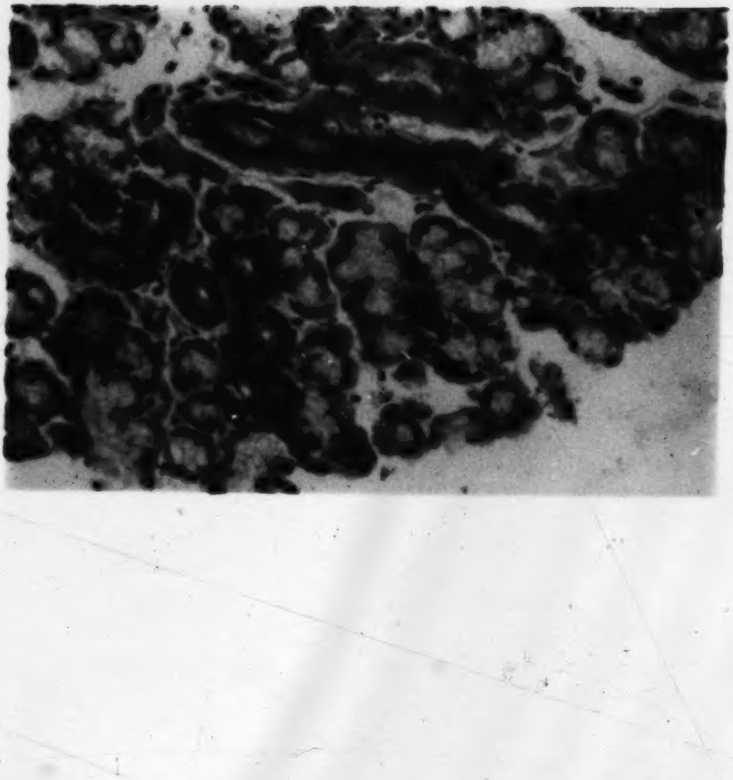


Plate XV : Localization of Kallikrein in Cat Sublingual Gland by Immunofluorescence Microscopy.

a) Tissue section after reaction with antibody to submandibular kallikrein. Section from main body of the gland, devoid of any striated ducts. Original magnification: 65X.

b) Tissue section after reaction with antibody to submandibular kallikrein. Section from peripheral lobules of the gland or from contaminating portion of submandibular gland (see plate XIV and text). Original magnification: 65X.

Plate XV.

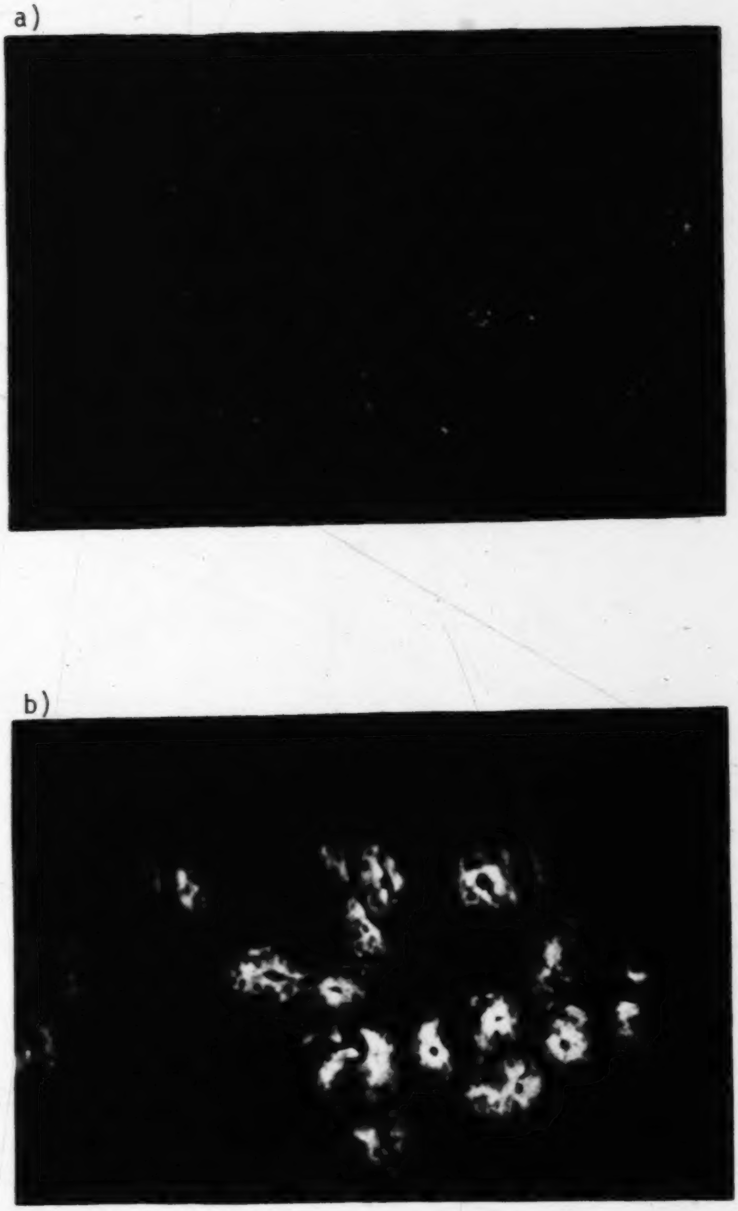
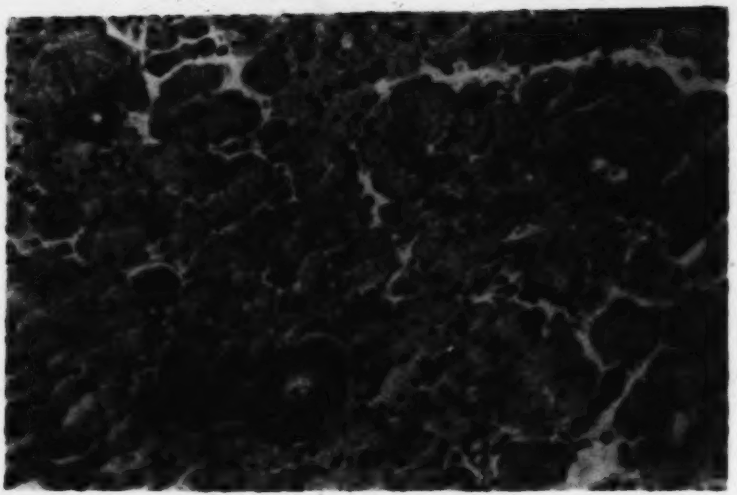


Plate XVI : Haematoxylin-Eosin Stained Sections of Cat Parotid Gland.

Note small number of striated ducts, three in this section, compared to submandibular gland (plate VII). Original magnification: 65X.

Plate XVI.



serum containing antibody to cat submandibular kallikrein. Fluorescence was found to be limited to striated duct cells and concentrated mostly at the apical portion of these cells. As shown in plate XVII, only faint auto-fluorescence was observed in other portions of the gland. The paucity of striated ducts in the parotid gland explains the low kallikrein content of this gland.

2.5 Kidney and pancreas.

No fluorescence, except some autofluorescence, was observed in these tissues using our immune serum to cat submandibular gland kallikrein. Activation of pancreatic kallikrein and/or other fixation procedures might have been necessary to get specific fluorescence in these glands. However, no such investigations were pursued.

2.6 Comparison of kallikrein in submandibular, parotid and sublingual glands.

2.6.1 Kallikrein content.

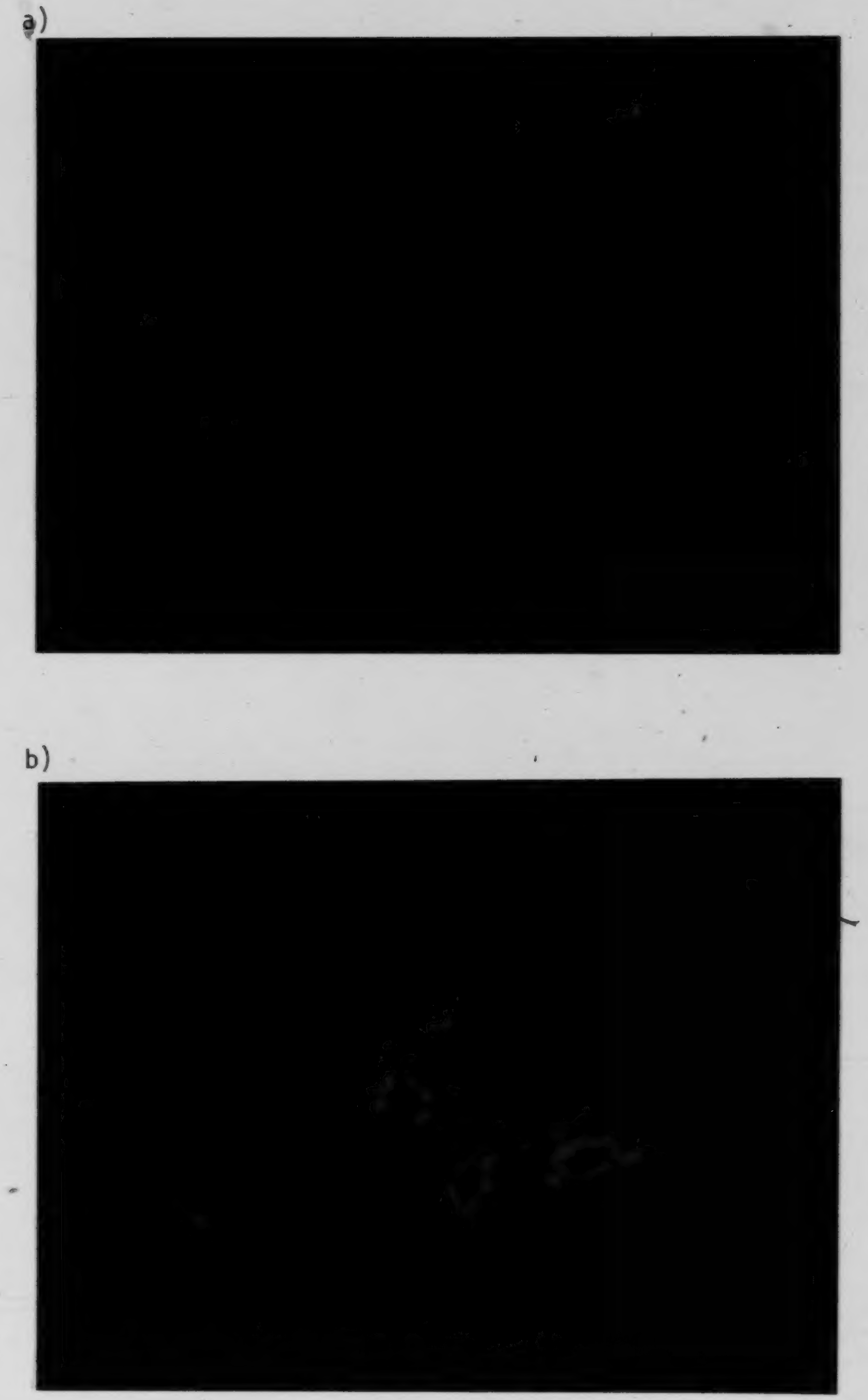
The kallikrein concentrations of freeze-dried glands were measured chemically in esterase units using BAEe as substrate (Trautschold, 1970). In one cat, the respective values for freeze-dried extracts of the submandibular, parotid and sublingual glands were 410, 18 and 45 mu/mg and 1273, 60 and 24 mu/mg for a second cat. These results fit within the variation found from cat to cat. The esterase activity determined in freeze-dried extracts of submandibular glands for example ranged from 379 mu/mg up to 2182 mu/mg for 10 cats studied. In general, the kallikrein content in these three salivary glands corresponded well to the number of striated and stratified collecting ducts observed, reinforcing the hypothesis that salivary kallikrein

Plate XVII : Localization of Kallikrein in Cat Parotid Gland by Immunofluorescence Microscopy.

a) Control tissue section without addition of antibody to kallikrein. There is only a slight and non-specific fluorescence throughout. Original magnification: 65X.

b) Tissue section after reaction with antibody to submandibular kallikrein. Note the characteristically few striated ducts and their moderate luminal fluorescence compared with a corresponding section of the submandibular gland (see plate XII). Original magnification: 65X.

Plate XVII.



is exclusively located in these cellular structures (plate XVIII)-

2.7 Stimulation and ligation experiments.

Immunofluorescence in the cat's submandibular gland was compared before and after nerve stimulation and/or duct ligation, procedures which are known to affect the kallikrein content (Beilenson et al., 1968; Barton et al., 1975) and the number of apical secretory granules of this gland (Schachter et al., 1977).

2.7.1 Kallikrein content.

As shown in fig. 16, the kallikrein esterase activities were variable but similar effect was produced by duct ligation and/or nerve stimulation, the kallikrein content of the treated glands being greatly reduced, if not completely abolished.

2.7.2 Immunofluorescence.

Plate XIX illustrates the decrease in specific fluorescence with the disappearance of kallikrein in ligated and/or sympathetic stimulated gland. No noticeable change in fluorescence was observed after chorda stimulation. These results are direct evidence for the relationship between sympathetic stimulation and kallikrein depletion in cat submandibular glands. A similar experiment, duct ligation, was also carried out in the cat parotid gland and similar results were obtained, with almost complete disappearance of specific fluorescence after a four day ligation (Plate XX).

2.8 Cat submandibular gland main excretory duct.

Duct portions were processed the usual way for immunofluorescence staining. As illustrated in plate XXI, specific fluorescence was observed in the apical portion of the cellular epithelium lining

Plate XVIII : Localization of Kallikrein in Cat Salivary Glands by Immunofluorescence Microscopy.

Note variation in intensity of specific fluorescence from gland to gland. Tissue sections after reaction with antibody to submandibular kallikrein.

a) Sublingual gland (Peripheral lobules)

b) Submandibular gland

c) Sublingual gland (inner lobules)

d) Parotid gland.

Original magnification: 65X.

7

Plate XVIII.

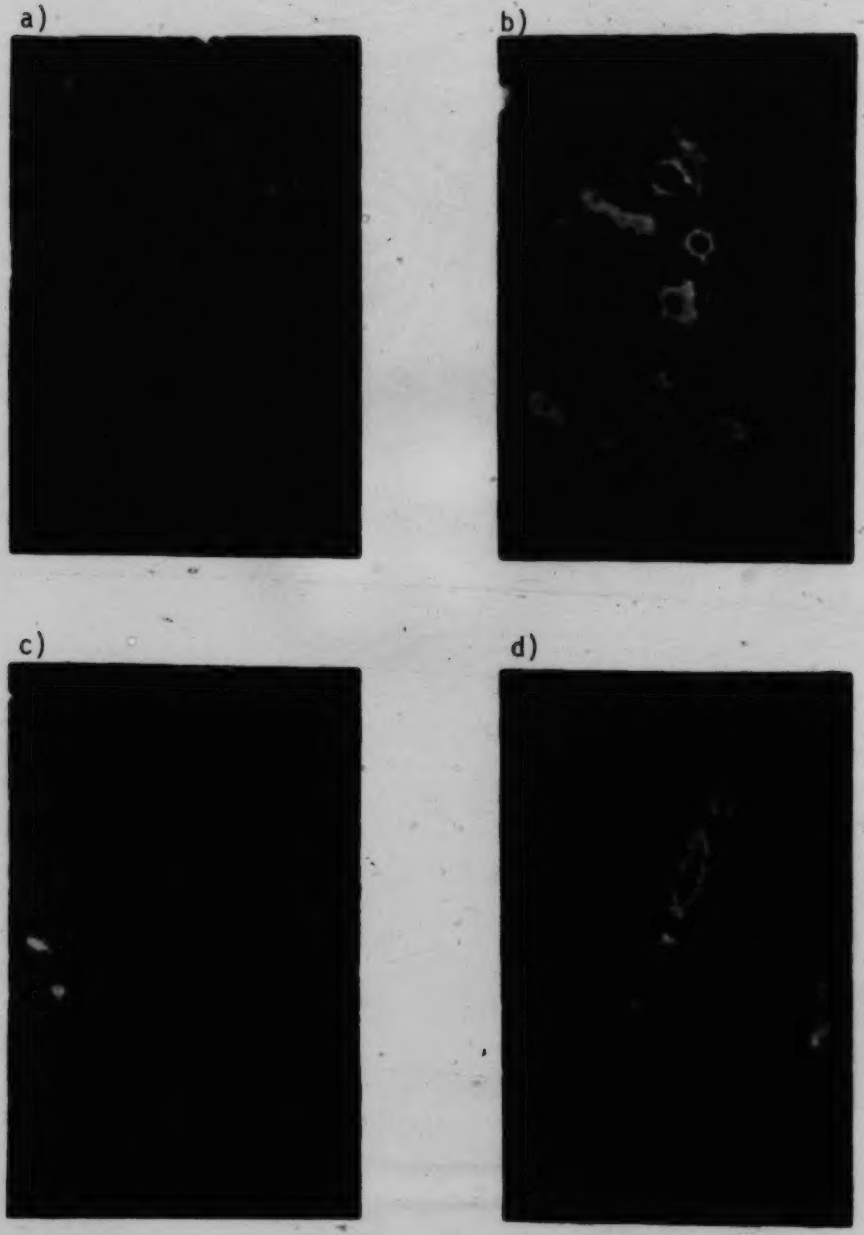


Fig. 16. Kallikrein Content in Nerve Stimulated and/or Ligated Submandibular Glands of the Cat.

Cat #	Treatment	Esterase Activity (mU/mg)	% of Activity (Experimental gland/control)
102176	Symp. stimul.	167.7	26.2%
	Control	640.4	
122976	Symp. stimul.	86.5	14.6%
	Control	590.9	
123176	Duct ligation & symp. stimul.	77.3	3.5%
	Control	2181.8	
032477	Duct ligation	13.6	1.4%
	Control	990.9	
020877	Chorda stimul.	413.6	109%
	Control	378.8	

Both ligated and/or stimulated submandibular glands and contralateral glands were excised after samples had been taken out for immunofluorescence staining. The glands were rinsed in 0.9 NaCl, minced, weighed, and freeze-dried. Aqueous extracts of the freeze-dried glands were centrifuged, the supernatant freeze-dried again and the resulting powder used for kallikrein esterase activity determination. This activity was measured chemically by the method of Trautschold (1970) using BAEe as substrate. Esterase activities are expressed in mU/mg of freeze-dried extract.

Plate XIX : Localization of Kallikrein in Cat Submandibular Gland by Immunofluorescence Microscopy after Nerve Stimulation and/or Duct ligation.

All pictures except d) are tissues sections after reaction with antibody to submandibular kallikrein.

a) Control section without nerve stimulation or duct ligation.

b) Section of gland whose kallikrein concentration had been reduced to 3% of the contralateral unstimulated gland by duct ligation for four days and subsequent sympathetic nerve stimulation. There is only diffuse, non-specific fluorescence.

c) Fluorescence in striated ducts after sympathetic stimulation. The kallikrein concentration was reduced to 15% of the contralateral unstimulated gland. Note the decreased intensity of duct fluorescence and its concentration near the lumen only after sympathetic stimulation.
Original magnifications of a), b) and c) : 65 X.

Plate XIX.

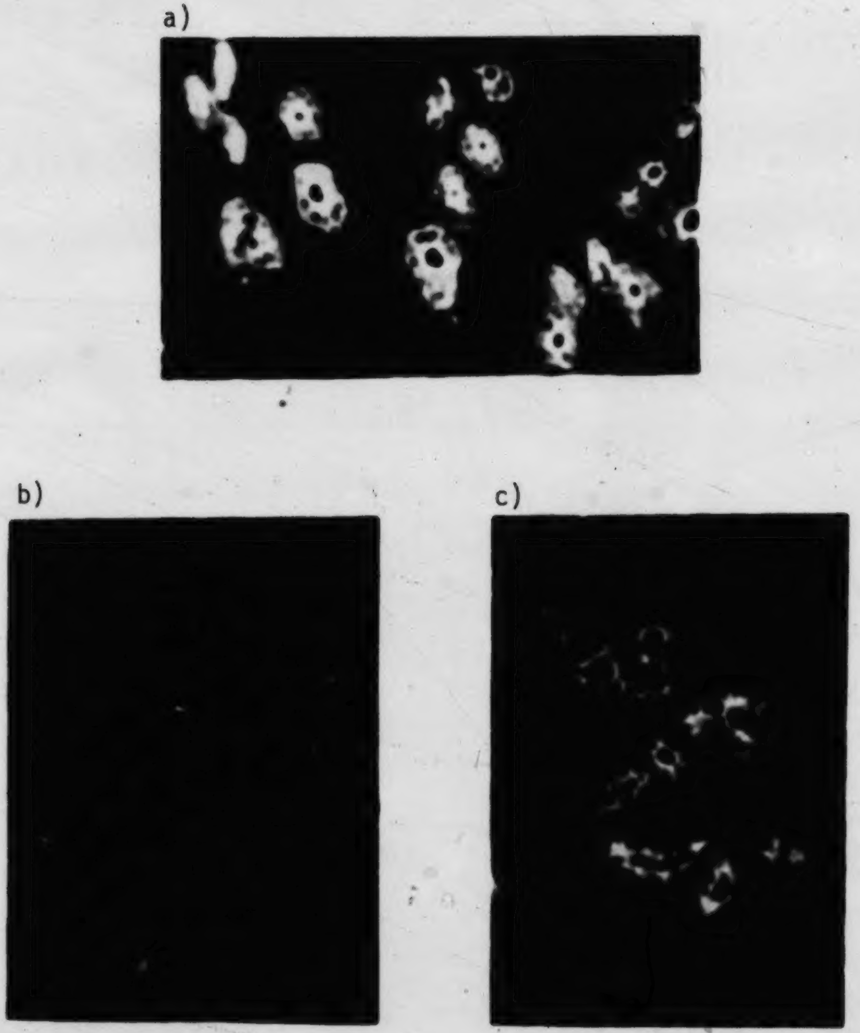


Plate XIX : Localization of Kallikrein in Cat Submandibular Gland by Immunofluorescence Microscopy after Nerve Stimulation and/or Duct Ligation.

d) Control tissue section without addition of antibody to kallikrein.

e) Section of gland after parasympathetic nerve stimulation. Note the intense specific fluorescence still present in the striated duct.

f) Control section without nerve stimulation or duct ligation.

Original magnification of d), e), and f) : 160X.

Plate XIX.

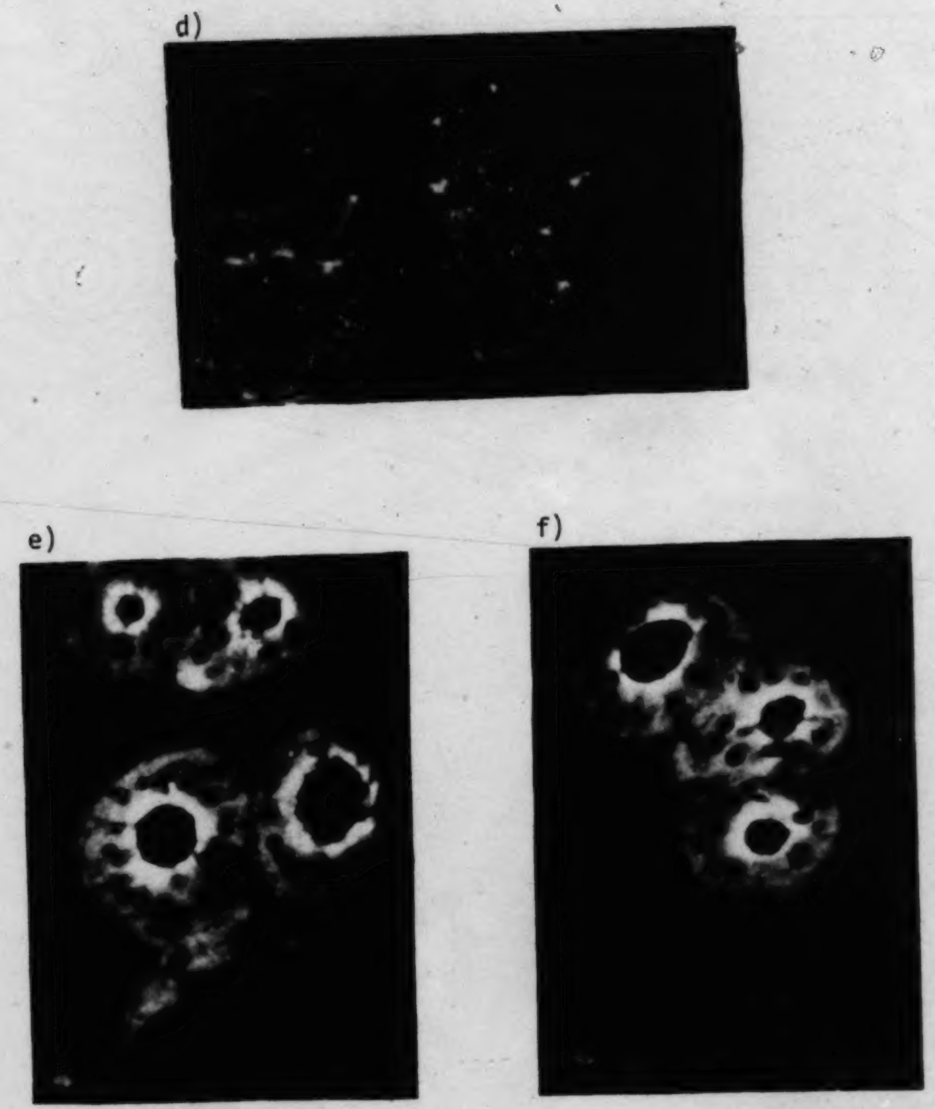


Plate XX : Localization of Kallikrein in Cat Parotid Gland by Immunofluorescence Microscopy after Duct Ligation.

Tissue sections after reaction with antibody to submandibular kallikrein.

a) Control section from unligated gland. Note specific fluorescence at the luminal portion of striated duct cells. Original magnification: 65X.

b) Section from ligated gland in which kallikrein content had been reduced to 4% of the contralateral unligated gland. There is no evidence of specific fluorescence. Original magnification: 65X.

Plate XX.

a)

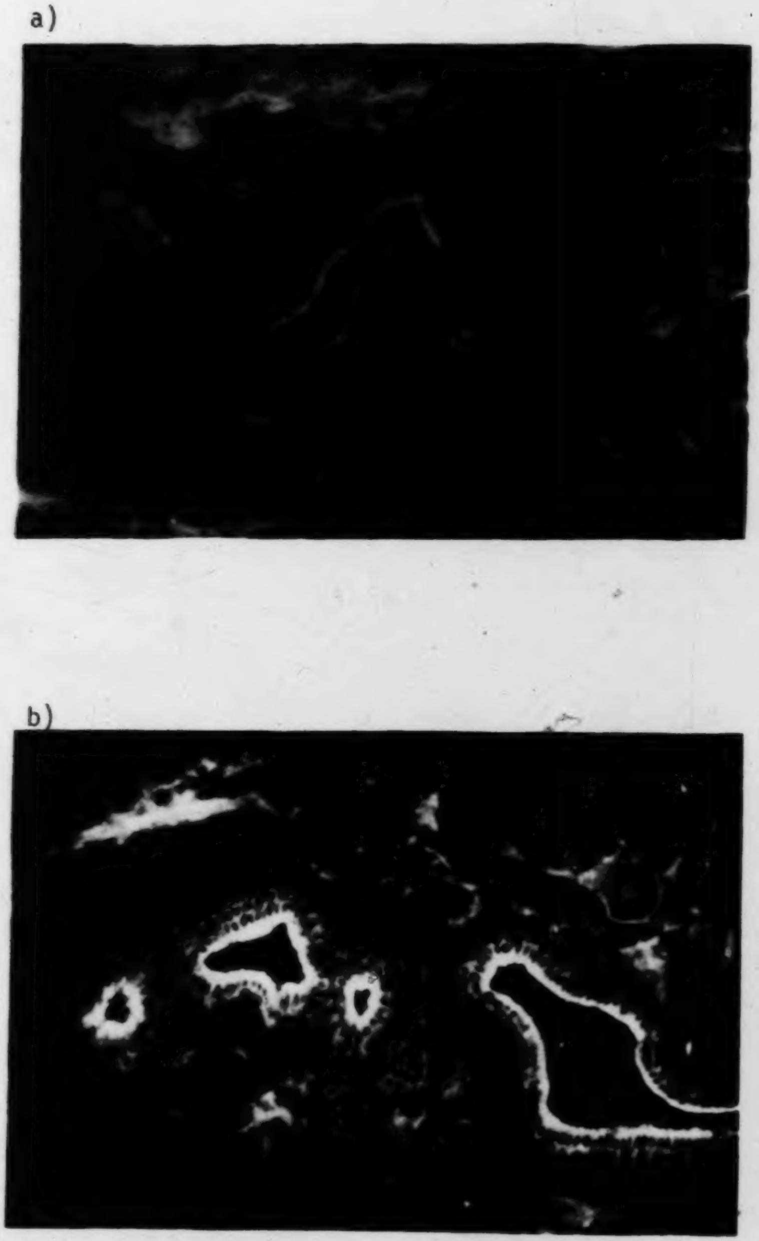
b)

Plate XXI : Localization of Kallikrein in Cat Main Excretory Duct by Immunofluorescence Microscopy.

a) Control tissue section without addition of antibody to kallikrein. Original magnification: 65X.

b) Tissue section after reaction with antibody to submandibular kallikrein. Note the specific fluorescence in the apical portion of the first layer of epithelium lining the lumen. Original magnification: 65X.

Plate XXI.



the lumen. No fluorescence was present in the surrounding structures. However, the results were not consistant from cat to cat, and in some experiments no fluorescence could be detected in the submandibular MED.

As shown in plate XXII, some secretory granules, similar to those observed in striated ducts, were found in a position corresponding to the fluorescence previously seen in wax sections stained for immunofluorescence.

3. Dog.

3.1 Dog renal kallikrein and its antibody.

As illustrated in fig. 17, no antibody was found to be present in the serum collected from one of the two rabbits injected with sample A antigen while clear precipitin bands were observed with the sera of the two other rabbits when tested by immunodiffusion versus sample A antigen. Surprisingly, no precipitin arcs formed when the sera were tested versus sample B DRK, which contained small amount of ammonium sulfate, and this might have hampered the results of the immunodiffusion test.

3.2 Localization of kallikrein in kidney.

No specific fluorescence could be observed in sections stained for immunofluorescence, the fluorescence being diffuse and/or present in both control and specifically stained sections. Special fixation techniques might have been necessary to preserve the antigenicity of the kallikrein present in the tissue as suggested by Orstavik et al., (1976). However, no further investigation was pursued.

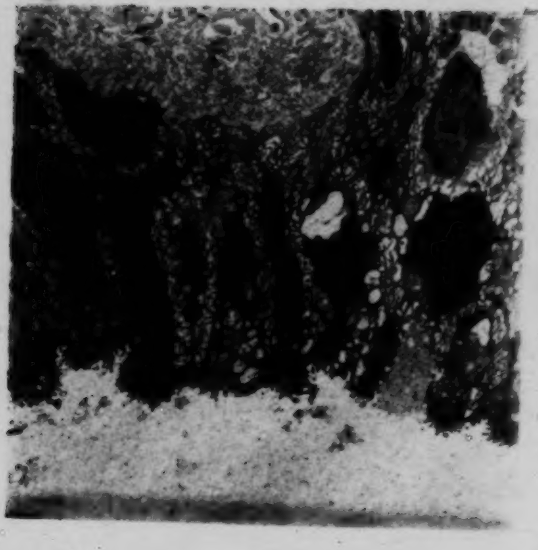
Plate XXII: Electron Micrographs of Luminal Portion of Cat Main Excretory Duct.

a) Cross-section of the main excretory duct showing the low number of light cells compared to dark ones. Original magnification: 924X.

b) Details of the luminal portion of a light cell from the main excretory duct. Note some secretory granules similar to those observed in striated (plate X) and stratified ducts (plate XI). Original magnification: 4729X.

Plate XXII.

a)



b)

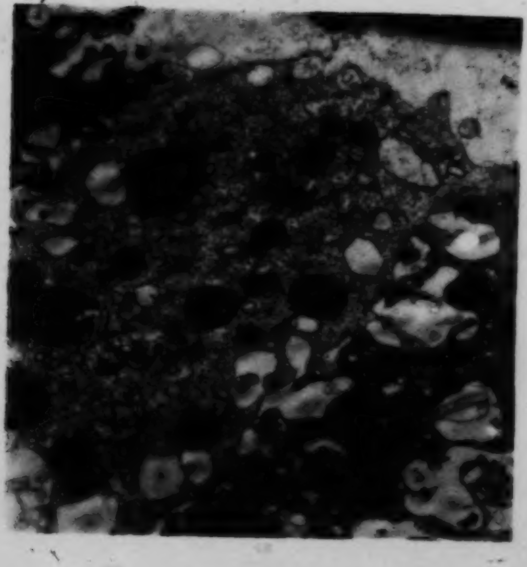
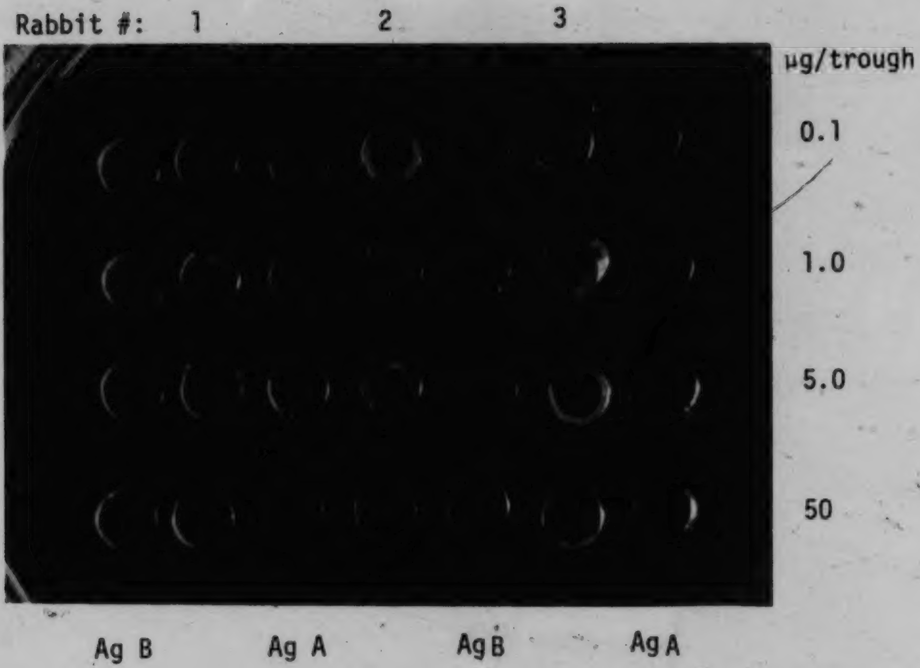


Fig. 17. Immunodiffusion of Dog Renal Kallikrein.

Sera from three rabbits injected with DRK were tested by immunodiffusion. Rabbit 1 and 2 were immunized with DRK sample A and rabbit 3 with DRK sample B. All sera were tested versus both sample A and B of antigen but reacted only with sample A. Numbers represent the total amount in µg of antigen per well. 35 µl of solutions was placed in each trough.

Fig. 17.


Rabbit #:
1
2
3
µg/trough
0.1
1.0
5.0
50
Ag B
Ag A
Ag B
Ag A

DISCUSSION

1. Review and Assessment of Results.

The main objective of the experiments described in this work was the localization of kallikrein in the submandibular gland of the cat. The work was undertaken first in the guinea-pig coagulating gland because coagulating gland kallikrein was made available to us and because the localization of CGK by Immunofluorescence was found to be feasible in that gland. Thereafter, the details of this immunocytochemical technique were developed and improved, using the guinea-pig's coagulating gland. There also seemed to be cross-antigenicity between coagulating gland kallikrein and submandibular gland kallikrein of the guinea-pig permitting us to also attempt to localize the enzyme in the submandibular gland of that animal.

When purified cat's submandibular gland kallikrein became available, the same series of experiments were performed on the cat submandibular, parotid and sublingual glands as well as on kidney and pancreas. Immunofluorescence was chosen as the method of localization because of its high specificity. Since the indirect immunofluorescence technique does not require pure antibody and results in higher fluorescence in the tissue due to additive effects of the two antibodies used, this technique was preferred over the direct technique. After some initial problems with excess antigen, resulting in the death of rabbits being immunized, antibody specific for kallikrein was obtained. Immunofluorescence staining was thus possible.

1.1 Guinea-pig.

Although the resolution obtained under fluorescence microscopy is not high enough to permit a precise subcellular localization of antigen, the diffuse specific fluorescence observed in the secretory cells lining the lumen of the coagulating gland is in accord with the previous suggestion (Barton and Schachter, 1973), based on differential centrifugation procedures, that CGK is present free in the cytosol of cells of these glands rather than in organelles. This observation was surprising since kallikrein in other tissues has always been assumed or concluded to be in secretory granules (Bhoola, 1970; Bhoola and Heap, 1970; Garrett and Kidd, 1975; Barton et al., 1975; Schachter et al., 1977). Immunocytochemical methods employing electron microscopy might thus have to be used to definitely confirm the cytosol location of kallikrein in the coagulating gland of the guinea-pig.

The present investigation with CGK permitted us also to attempt to localize kallikrein in the submandibular gland of the guinea-pig using antibody specific for CGK. This was possible since it was found that submandibular kallikrein and CGK were immunologically cross-reactive with one another, in the same way as urinary and salivary kallikrein (Brandtzaeg et al., 1976). In the submandibular gland, kallikrein was localized in the apical portion of duct cells only, supporting some earlier indirect evidence resulting from electron microscope studies in the cat submandibular gland (Garrett and Kidd, 1975; Schachter et al., 1977). Kallikrein has recently also been localized by immunofluorescence technique in the apical border of cells in the granular and striated ducts of the salivary glands of the rat

(Orstavik et al., 1975; Brandtzaeg et al., 1976) and in the striated duct of the pig (Dietl et al., 1978). In all these investigations, there was no evidence of kallikrein in other cell types, e.g. in acinar or demi-lune cells.

Bhoola and co-workers (see Bhoola, 1970; Bhoola and Heap, 1970) concluded that there was acinar localization of kallikrein in submandibular gland of various mammals. The latter conclusion was arrived at indirectly by isolating different populations of organelles from tissue homogenates and by making inferences about the localization of the enzymes in the intact gland from the appearance and kallikrein content of populations of isolated organelles. Our results, along with the other previously mentioned investigations, do not support Bhoola's conclusions.

The exclusive localization of kallikrein in the duct system of the guinea-pig's submandibular gland was supported by duct ligation experiments which resulted in almost complete depletion of kallikrein and disappearance of specific fluorescence in ligated glands. The prostate gland of the guinea-pig was also found to contain small amounts of kallikrein but the localization of this enzyme by immunofluorescence in that tissue was not successful. This might be explained by the low kallikrein concentration in the prostate or by the lack of immunological cross-reactivity between prostate and coagulating gland kallikrein.

An attempt to localize acrosin in guinea-pig sperm by using antibodies specific for CGK was also negative. Further investigations are thus necessary to localize these kininogenases and to asset their difference or similarity in immunological cross-reactivity.

Castration experiments in the guinea-pig did not seem to produce a marked effect in the content or localization of kallikrein in coagulating and submandibular glands.

1.2 Cat.

The localization of kallikrein in the cat's submandibular gland was our main objective since most of the preceeding investigations completed by Schachter and co-workers had been carried out in this particular tissue (Beilenson et al., 1968; Karpinski, et al., 1971; Barton et al., 1975; Schachter et al., 1977). Also, many studied have dealt with the possible rôle of salivary kallikrein in functional hyperaemia in this same organ (Hilton and Lewis, 1955; Bhoola et al., 1965; Karpinski et al., 1971).

Specific fluorescence, indicative of the presence of kallikrein, was observed in the apical region of the striated ducts particularly, but also in the luminal epithelial cells of the stratified epithelial collecting ducts. Fluorescence was not seen in other glandular components, viz., acinar, demi-lune or myoepithelial cells, or interstitium. This supports an exclusive location of kallikrein in the ductal system of the submandibular gland of many mammals. Our conclusion was supported by nerve stimulation and/or duct ligation procedures in which kallikrein depletion parallelled the disappearance of fluorescence from the tissue. Moreover, sympathetic stimulation resulted not only in a reduction in intensity of specific fluorescence but also in its increased localization towards the lumen. There was no evidence of secretion of kallikrein either into or towards the interstitium of the gland. The concentration of specific fluorescence near the apical region of the striated duct cells indicates that secretion of kallikrein is into the lumen of the duct and apparently exclusively so. Also, after parasympathetic nerve stimulation accompanied by copious secretion, there was again no sign of any movement of the fluorescence towards the basal portion of the cell, which might be expected if kallikrein entered the interstitial tissue to release kinin and cause functional vasodilatation.

The results obtained by immunofluorescence were supported by electron microscope studies. Sympathetic nerve stimulation and/or duct ligation have been shown to reduce the kallikrein content of the cat's submandibular gland (Barton et al., 1975) and also to deplete the striated ducts of their small apical secretory granules which have been postulated to be the site of kallikrein storage (Schachter et al., 1977). The location of these small secretory granules (0.2 - 0.5 μm) corresponded well to the fluorescence observed in tissue sections and their depletion also parallelled the disappearance of the fluorescence after sympathetic nerve stimulation nerve stimulation and/or duct ligation procedures.

Localization studies were also extended to the larger stratified epithelial collecting and main excretory ducts. Specific fluorescence was observed at the luminal border in almost all collecting ducts while the results were variable for the excretory duct, occasionally showing specific fluorescence which was confined to the luminal epithelial cells. The specific fluorescence in those larger ducts was also decreased after sympathetic nerve stimulation.

Under the electron microscope, small secretory granules, similar to the ones observed in the striated duct cells, were also found in the larger ducts, and occasionally in the main excretory duct. The granules in these ducts were present only in the luminal layer of stratified epithelial cells. Like those of the striated ducts, the granules were apically located, varied somewhat in diameter and density and appeared to be secreted into the lumen by exocytosis.

The general impression gained from the fluorescence in the duct system is that there is decreasing concentration of kallikrein from the striated ducts to the main excretory duct. Electron microscope observations also suggested that concentration of granules also decreased in number with increase in duct size.

Immunofluorescence studies were also performed in the parotid and sublingual glands, using antibody for submandibular kallikrein. Again, kallikrein was exclusively located in the striated and collecting ducts of the parotid gland. The sublingual gland differed from the parotid in that most sections failed to show any specific fluorescence at all. Occasionally, a section was found which contained may striated ducts which showed considerable fluorescence. However, if a similar section from a same series of serial sections was stained for light microscopy observation, it was observed that the general appearance of the tissue was very similar to the submandibular gland, containing large acinar cells capped with demi-lune cells and a great number of striated ducts. This observation was not consistent from cat to cat, suggesting that a portion of the submandibular gland might have been dissected out along with the sublingual gland (which lies along the submandibular gland) or that some of the lobules, apparently peripheral ones, have a structure completely different from the inner lobules. Further examination of the sublingual gland is necessary to definitely answer this question.

Since kallikrein appears to be exclusively located in striated and collecting ducts, and since these ducts are abundant in the submandibular but rare in the parotid and almost absent in the sublingual glands, this explains the correlation between specific fluorescence observed in these glands and their kallikrein content.

Hoping that there would be immunological cross-reactivity between the cat pancreatic and/or renal kallikrein with the cat submandibular kallikrein, immunofluorescence staining was attempted on pancreas and kidney sections using antibody to submandibular kallikrein. However, specific fluorescence was not observed in either tissue. This could be explained by the lack of immunological cross-reactivity between these kallikreins or by the fixation technique that had to be used for the rat kidney (Orstavik et al., 1976) and the pig pancreas; (Dietl et al., 1978). The same might therefore be true for these organs in cat.

1.3 Dog.

An attempt to localize kallikrein in the kidney of the dog was unsuccessful. Unfortunately, the immunofluorescence technique proved to be inadequate in this instance; the same results were obtained in the control and antibody treated sections. Some fluorescence was occasionally seen in the interstitium around the tubular cells and the glomeruli suggesting that the antibody raised might have been specific to another protein like collagen rather than to kallikrein. This is reinforced by recent investigations in our laboratory showing also interstitial fluorescence in dog's submandibular gland using dog renal kallikrein antibody. However, this does not exclude the possibility that fixation procedures might also have been unsuitable for the dog's kidney.

2. Implications of results: Physiological rôles of kallikrein.

Since no physiological rôle is known yet for the kallikreins it was hoped that this investigation might help to define such a rôle or to confirm some hypotheses which had been suggested.

2.1 Functional hyperaemia.

Some authors, Hilton and Lewis in particular (see Hilton, 1970), believe that functional vasodilatation is produced by the action of kallikreins and the kinins they release. Others (see Schachter, 1970), however, suggest that functional hyperaemia is caused by parasympathetic vasodilator nerves. The present results confirm earlier statements (Beilenson et al., 1968; Barton et al., 1975; Schachter et al., 1977) that kallikrein is depleted from submandibular striated duct cells by sympathetic nerve stimulation and/or ligation of the main excretory duct while no significant reduction is produced by parasympathetic stimulation. The conclusion at which Hilton and co-workers arrived, i.e. that true parasympathetic vasodilator nerve fibers do not exist and that the vasodilatation accompanying parasympathetic nerve stimulation is due to kallikrein released from secretory cells, is thus challenged again by our present results. It was found again that parasympathetic stimulation had no significant effect on the kallikrein content of the gland (despite copious secretion of saliva), but also that the fluorescence was unaffected. On the contrary, sympathetic stimulation resulted in a reduction in kallikrein concentration and in the concentration of fluorescence at the apical portion of the striated duct cells. The latter observation suggests that kallikrein is exclusively secreted into the lumen of the duct. It would be surprising if kallikrein, secreted into the duct, could find its way back to the blood vessels, release kinin, and then cause a vasodilatation to begin with a latency of only 350 m.sec. such as occurs after stimulation of the chorda lingual nerve (Karpinski et al., 1971). Moreover, it is unlikely that kallikrein has a major rôle in functional vasodilatation in parotid and sublingual glands since it is almost absent from these glands. However, Nustad and co-workers (see Nustad et al., 1978) suggest that there is intraglandular diffusion of kallikrein in the cat submandibular gland. Their evidence was based on intraductal and subcapsular application of radioactive labelled kallikrein which penetrated the duct walls and entered the gland circulation. They concluded that endogenously released kallikrein will have access and might influence the gland microcirculation. They finally suggest that vasodilatation following parasympathetic nerve stimulation is initiated by vasodilator nerves but sustained and augmented by kinin. Their hypothesis is plausible but does not explain the high release of kallikrein after sympathetic compared to parasympathetic stimulation. Further details of their investigation, to be soon published, are needed to thoroughly discuss their findings. A definite answer to the implication of kallikrein in vasodilatation might eventually be found by the ultrastructural localization of kallikrein after parasympathetic and sympathetic stimulation.

2.2 Membrane permeability and electrolyte transport.

From our results and from preceeding investigations (Barton et al., 1975; Garret and Kidd, 1975; Schachter et al., 1977) it appears that kallikrein is apparently exclusively secreted into the lumen of the duct system of the cat's submandibular gland. Some of the physiological possibilities therefore are that salivary kallikrein may play a rôle in sodium transport in the ducts, as a digestive enzyme, in absorption, or in cell proliferation and repair in the digestive tract (see Schachter & Barton, in Press).

It had been suggested that kidney kallikrein may have a rôle as a natriuretic hormone corresponding to the sodium-retaining rôle of renin (Carretero and Scicli, 1976). Since renin-like enzymes have been observed in the submandibular glands of many mammals (Bhoola, Dorey and Jones, 1973; Gutman, Levy and Schorr, 1973; Garcia, Boucher and Genest, 1976), are secreted in high concentrations in the saliva and are probably located in the secretory granules of the convoluted or striated ducts (Cozzari, Angeletti, Lazor, Orth and Gross, 1973; Michelakis, Yoshida, Menzie, Murakami and Inagami, 1974), a further parallel can thus be drawn between kallikrein and renin-like enzymes. The suggested sodium-retaining rôle of kallikrein in kidney might thus be extended to the salivary glands, where sodium would be selectively reabsorbed in the duct system, resulting in hypotonic saliva. This suggestion is reinforced by our results in the cat's sublingual gland. Sublingual saliva is unusual in being isotonic (Burgen and Emmelin, 1961) and our results indicate that very few, if any, kallikrein containing ducts exist in that gland. The lack of kallikrein might thus explain the high sodium content of sublingual saliva. Also, there might be a correlation between secretion of potassium and kallikrein concentrations. The secretion of potassium in the kidney takes place at the distal tubular cell level (Schneyer et al., 1972), cells which have been shown to contain kallikrein (Orstavik et al., 1976). Morover, kallikrein release is influenced by aldosterone (Young et al., 1976) which also regulates potassium secretion. Since kallikrein is found in the duct systems of salivary glands, ducts which appears to be the site of potassium secretion into saliva (Schneyer et al., 1972), having a rôle similar to the distal tubules in the kidney, it is thus possible that kallikrein is directly or indirectly involved in potassium secretion .

Other rôles of salivary kallikrein, such as increasing cell proliferation or repair of damaged cells in the digestive tract, or simply as a digestive enzyme, are also possible. Further investigations are necessary to define these rôles.

2.3 Other rôles and general considerations.

Recent observations by Schill and co-workers (Schill and Haberland, 1974; Schill, 1975), suggest a possible rôle for kallikrein in the coagulating or other sex glands. They found that pancreatic kallikrein increased sperm motility (*in vitro*) and the sperm count of ejaculates after the administration *in vivo*. Another possible rôle of kallikrein in reproductive physiology might be to help control tonicity of the seminal fluid by selective reabsorption of sodium or secretion of potassium. Again further investigations are needed before assessing a precise function to this kallikrein.

There is fairly strong evidence from all the tissues described up to now (kidney, pancreas, salivary and coagulating glands, and others) that kallikrein is located in exocrine cells and that its rôle is probably extraglandular. Only the implication of kallikrein in electrolyte transport, sodium in particular, would appear to be applicable to most systems but this rôle has yet to be characterized and confirmed in even one tissue before being generalized.

Since kallikreins belong to the wider group of serine proteases such as trypsin, thrombin, fibrinolysin and elastase (Stroud, 1974), they may have diverse physiological rôles related to the tissue in which they are located rather than having a common rôle in all these systems. The particular function of kallikrein in each tissue might have evolved with their diversification from a common origin since all serine proteases have a common and a very specific configuration of amino acids at their active site. This common configuration of amino acids suggests that, at the origin, one gene was coding for a protein having that same sequence and that, during the evolution process, mutations slowly affected that original gene. New and closely resembling proteins were then coded by the resultant genes and appeared in various tissues of the same or different species, but differed in their physiological functions.

On the other hand, it has been demonstrated that the duct cells of salivary glands of various mammals are the site of many biologically active proteins, like epidermal and nerve-growth factors, renin-like enzymes, sialotomin, lysozyme and various esteroproteases (see Schachter et al., 1978). This tends to indicate that the salivary glands may have a greater physiological significance than has been believed up to now.

3. General conclusion.

The localization of kallikrein in secretory cells of the guinea-pig's coagulating, and submandibular glands and of the cat's salivary glands, suggests rôles linked to kallikrein secretion in the duct system. These rôles might be linked to electrolyte transport, tissue repair, cell proliferation and/or sperm motility but careful characterization of such kallikrein functions are needed before these hypotheses can be verified.

Immunocytochemical methods employing electron microscopy for the ultrastructural localization of kallikrein in tissue will probably bring answers to many of the unsolved questions regarding the physiological significance of these important enzymes that are the kallikreins.

It appears that the kallikrein-kinin system has to be investigated further before definite conclusions can be drawn about its rôle and implications in biological activities.

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